PRIFYSGOL BANGOR / BANGOR UNIVERSITY

PRIFYSGOL
BANGOR
UNIVERSITY

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

Baker, Paul; Charlton, Adam

Peer reviewed version

Cyswllt i'r cyhoeddiad / Link to publication

11. May. 2021

1 **A comparison in protein extraction from four major crop residues in Europe using**

2 **chemical and enzymatic processes**

3

4 Paul W. Baker[*], Adam Charlton

5

6 Email: paul.baker@bangor.ac.uk

7 Tel: 0044 (0)1248-382540

8 Paul Baker (*) · Adam Charlton

9 BioComposites, Bangor University, Deiniol Road, Bangor, Gwynedd LL57 2UW, UK

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

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,

rapeseeds, tomatoes, and citrus fruits, where each comprise the bulk of crop waste within each of

their specific categories (Fig. 1).   Within the olive industry two phase or three phase extraction

of the oil is generally deployed, resulting in the formation of olive mill cake and olive mill waste

water (Souilem et al., 2017). Smaller companies may also perform destoning in order to produce

oils containing higher proportions of polyphenolics. The waste generated from the mills is

disposed on land but this can have a serious impact requiring soil remediation (Doula et al.,

2017). In contrast, rapeseed meal is often used to supplement ruminant feeds at 20-30%,

providing 50% of the animals' protein requirements, although significant variations in total

protein content may be caused by many different factors (Dale, 1996). Protein variability will

affect the market price of this material as an animal feed, but the presence of anti-nutritional

factors has affected its uptake in the wider animal nutrition sector, because glucosinolates and

phytates can make it unpalatable (Dale, 1996) although some cultivars have been selectively

breed which contain much lower quantities of anti-nutrients (Ghodsvali, Khodaparast, Vosoughi,

& Diosady, 2005). Citrus waste may also be used as animal feed after being dried and pelletized,

although the majority is discharged to landfill due to the high cost of drying (Negro, Mancini,

Ruggeri & Fino, 2016). However, new EU legislation requires that some attempt must be made

to valorise the waste before landfill disposal, which could include limonene extraction and the

production of biofuels. Likewise, only a small proportion of tomato waste may be used as

animal feeds or as organic fertilizer, but much of it is discarded as landfill waste due to the short

shelf-life of the tomatoes of less than one week (Fritsch et al., 2017). Consequently, methods are

underway to develop the recovery of a range of bioactives from tomato waste.

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 bioavailablity. 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^3 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

7

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-mercepatoethanol (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 trichloracetone 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 dithiothrietiol 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 (Kozlowska, 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 glucoinsolates 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 acheived 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 μg/ 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 μg/ kg and 3 μg/ 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

16

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 α-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 mean (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 ß-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

22

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

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-

608   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 Aydınoğlu, T., & Sargın, 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   solvatation 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., Ribaillier, 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 Kozlowska, 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., & Lecomt, 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.

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

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

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

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

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

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

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

Rehder, A., Sulewska, A. M., Markedal, K. E., Sørensen, S., & Sørensen, J. C. (2017). Solubility of a cruciferin-rich protein product purified from rapeseed pressed cake (*Brassica napus* L.) by an aqueous processing method. *International Journal of Food Science and 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 Savadkoohi, 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 Scherhaufer, 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.

Table 1

Sample	Enzyme	Protein	Reference
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