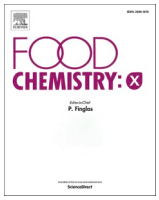
Food Chemistry: X 19 (2023) 100819

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Oil and protein extraction from fruit seed and kernel by-products using a one pot enzymatic-assisted mild extraction

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ARTICLE INFO

Keywords:
Fruit processing waste
Seeds
Kernels
Enzymatic-assisted extraction
Oil
Protein

ABSTRACT

This research evaluated the application of a one-pot enzymatic extraction by using a protease for the concomitant and sustainable extraction of oils and proteins from fruit seeds/kernels of different species of stone, citrus and exotic fruits.

The proteolysis improved the oil solvent-extractability of seeds/kernels of some fruit species compared to the use of acid and/or organic solvents and led to directly recover fat (10–33%) from mango, lemon and pumpkin seeds. Good protein extraction yields were obtained compared to conventional solvent extractions and with a good hydrolysis degree (almost 10%) in the case of lemon and pumpkin seed protein hydrolysates. The nutritional quality of all the protein hydrolysates was quite low, because of their limiting amino acids (histidine, methionine and lysine). On the contrary, the fruit seed/kernel oils resulted with high nutritional value, as they were mostly rich in unsaturated fatty acids, primarily oleic acid (>25%) and linoleic acid (till 40%).

1. Introduction

The residual biomasses deriving from the food chain are by far those generating the largest amount of co-products, residues and wastes (about 1.3 BT/year) (FAO, 2011). For instance, fruit and vegetable processing sector generates about 90 million tons of by-products in Europe every year (Montenegro-Landívar et al., 2021). This decreases the existing capacity for waste management and resource depletion with negative effects on ecosystems (Jiang, Liao & Charcosset, 2020). In addition, the growing consumer awareness about the impact of food processing on the environment, sustainable diets and minimally processed foods, pushes companies to food innovation and to optimize processes in terms of energy consumption, carbon-footprint, and waste valorisation.

In this line, a bio-refinery strategy has been suggested to increase the overall resource efficiency of residual fruit biomasses and to produce high added value products (Kosseva & Webb, 2020). However, current applications and commercialization of fruit waste biorefineries are limited, mainly due to the lack of information on feedstock availability, process design, scale-up and the presence of potentially toxic substances in some kernels, as for example the cyanogenic glycoside amygdalin (D-mandelonitrile-β-D-gentiobioside), naturally present in some fruits of *Prunus genus* (i.e., apricot, peach, cherry, plum) (Bolarinwa, Orfila & Morgan 2014).

Nevertheless, fruit seeds and kernels represent an ideal substrate to be valorised, being naturally rich in nutrients (e.g., lipids, proteins etc.) and secondary metabolites (phytochemicals) that promote plant growth, germination and defence (Özcan, Ünver & Arslan, 2014).

Fruit seed oils are rich in unsaturated fatty acids (UFAs), but different fruits have specificity in fatty acid (FA) composition, thus giving the opportunity to cover a wide spectrum of different applications. For example, oils from the *Citrus genus* are mostly rich in palmitic acid (Anwar, Naseer, Bhanger, Ashraf, Talpur, & Aladedunye, 2008), suggesting they could be good candidates for palm oil substitution in specific food applications (i.e., bakery products, infant formula) or as raw material for oleo chemistry processes. Other fruit seeds and kernels can provide oils with high nutritional value, as they are rich in oleic (such as mango seeds, and apricot and cherry kernels) (Anwar, Rasul & Ashwath,

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https://doi.org/10.1016/j.fochx.2023.100819
Received 19 April 2023; Received in revised form 16 June 2023; Accepted 29 July 2023
Available online 31 July 2023

2019) or in linoleic acids (such as apple, pear and watermelon seeds) (Yukui, Wenya, Rashid & Qing, 2009).

The projected global demand for food proteins is largely exceeding current production capacities and it is expected to increase by 76% in 2050 (European Union – FOOD, 2030), due to the growing world population and the increasing demand for animal proteins. So, a possible technological option to enhance the sustainability of future protein supply is to increase the use of vegetal protein ingredients.

Finally, beside the possibility to explore a wide range of functional and bioactive chemicals with different applications, there is a growing need to study and develop environment-friendly processes, aimed to replace the conventional solvent-based systems. In this contest, green solvents, and technologies, like aqueous assisted enzymatic extraction, have demonstrated a great potential for the simultaneous oil and protein extraction from oilseed, preserving the quality of the extracted nutrients (Banerjee, Singh, Ranganathan, Macfarlane, Patti & Arora, 2017). Moreover, the advantages of the enzyme technology include the reduction of solvent and energy consumption, as well as the presence of other emerging process contaminants.

This study addresses the valorisation of fruit biomasses and the application of mild enzyme technology integrated with biorefinery cascade processing, as a more sustainable and eco-friendly method to simultaneously recover oil and proteins from fruit seed and kernel waste. To this aim, fruit residues covering different fruit varieties (e.g., stone fruits, citrus and exotic fruits) were characterized for their proximate composition, then an enzymatic assisted extraction protocol, based on the use of a protease and previously tested for other fruit by-products (Fuso, Viscusi, Larocca, Sangari, Lolli & Caligiani, 2023) was performed. Also, the evaluation of yields and chemical characterization of the oils (fatty acid and unsaponifiable composition) and proteins (amino acid profile and hydrolysis degree) resulting from the extraction and purification processes has been described.

2. Material and methods

2.1. Materials and chemicals

The fruit seeds/kernels from pumpkin (*Cucurbita pepo*) and exotic fruits (mango, *Mangifera indica*; avocado, *Persea americana*; litchi, *Litchi chinensis*; papaya, *Carica papaya*) were provided by SO.G.I.S. Industria Chimica S.p.A. (Sospiro (CR), Italy). Samples of lemon (*Citrus limon*) and stone fruit of genus *Prunus* (cherry, *Prunus cerasus*; peach, *Prunus persica*; apricot, *Prunus armeniaca*) were purchased from local market (Parma, Italy). Frozen seeds/kernels were milled with IKA A10 laboratory grinder and kept frozen at −20 °C until use for analysis.

The AccQ-Fluor reagent kit and AccQ-Tag^{TM} were obtained from Waters (Milford, MA, USA) and Amino Acid Standard H solution was purchased from Thermo Fisher (Thermo Fisher Scientific, Waltham, MA, USA). Kjeldahl defoamers and catalysts, DL-norleucine, Supelco 37 component FAME mix, 5-alfa-Cholestan-3-beta-ol, protease from *Bacillus licheniformis* (2.4 U/g, EC 3.4.21.62) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other solvents, salts, acids and bases were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Carlo Erba (Milan, Italy).

2.2. Proximate composition

The proximate composition analysis of each substrate was performed in triplicates by using the methods of AOAC International for vegetable matrices, slightly adapted (Fuso et al., 2023). Briefly, moisture was determined according to AOAC 925.09 by weighing the sample before and after drying in oven at 105 °C for 24 h and determining the difference. Crude ash was determined by gravimetric method after mineralization at 550 °C for 5 h + 5 h (AOAC 923.03). Total nitrogen was carried out according to 984.13 of the AOAC International with a Kjeldahl system (DKL heating digestor and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA). From the total nitrogen determined, protein percentage was calculated using 6.25 as nitrogen-to-protein conversion factor.

Crude fat content was determined using an automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) and made by immersion of the sample in the boiling solvent (diethyl ether) for 60 min, followed by a washing phase for 30 min (according to AOAC Method 920.39). Before lipid extraction, an acid pre-treatment was also formulated according to AOAC Method 954.02 with adaptations aimed to improve the oil extraction efficiency: 1 g of grinded sample was added to 20 mL of 4 N HCl solution and heated at 100 °C for 1 h. Then, the acid hydrolysed sample was filtered and rinsed with water till neutral pH. The filtered sample was dried in oven at 40 °C and subjected to the automatic extraction described above. The Soxhlet ether extracts were evaporated under vacuum at 40 °C and stored at −20 °C till further analysis.

Finally, total dietary fibres (TDF) were determined by enzymatic–-gravimetric official method AOAC 991.43. Digestible carbohydrates were determined by difference.

2.3. Enzyme-assisted extraction protocol

For the enzyme-assisted extraction (EAE) of nutrients from each fruit seeds/kernels, an extraction protocol previously published (Fuso et al., 2023) based on *Bacillus licheniformis* protease (the enzyme used in the AOAC 991.43 official method for dietary fibre determination), was performed. Briefly, an enzyme/substrate ratio of 1:100 (w/w) was mixed with a phosphate buffer solution (10 mM Na_2HPO_4/NaH_2PO_4) and hydrolysed at 60 °C and pH 7.5 overnight (16 h), then heated at 90 °C for 10 min to inactivate the enzyme. After centrifugation, two or three distinct phases were obtained, depending on the hydrolysed fruit seeds/kernels, from top to bottom: (i) free oil fraction (only for mango, lemon and pumpkin seeds), (ii) the aqueous fraction (supernatant), and (iii) the residual solid fraction (pellet).

After proteolysis, further analysis was performed in terms of total lipid yield (Eq. (3), paragraph 2.6) and molecular characterization, especially focusing on the lipid (see paragraph 2.4) and protein fractions (see paragraph 2.5). To this aim, most of the top surfaced oil (when present) was directly recovered, followed by a hexane wash of the supernatant (aqueous fraction) to ensure that oil was totally removed for accurate quantification. Also, the solid residue (pellet) was extracted by using conventional Soxhlet method to calculate total oil yield. Then, the enzymatically extracted lipids were characterized by GC–MS in terms of fatty acid and unsaponifiable fraction analysis, as described in paragraph 2.4, and compared with the lipid composition of acid/organic solvent-extracted fruit oils.

No residual oil was found in the aqueous phase that contained only proteins (in the hydrolysed form) and soluble fibres. So, proteins were purified from the soluble fibres by adding 95% v/v ethanol and by centrifugation (3900 rpm, 4 °C, 30 min). Then, the supernatant was recovered, lyophilized, and analysed for amino acid profile by UPLC-ESI/MS (see paragraph 2.5.1) and hydrolysis degree (see paragraph 2.5.2).

The overall experiment is represented in Fig. 1.

2.4. Molecular characterization of fruit oil by GC–MS

2.4.1. Fatty acid profile

Fruit oil samples were subjected to basic transmethylation according to ISO 12966-2:2017, slightly adapted. Briefly, 100 mg of fat were dissolved in hexane (5 mL) containing 0.2 mg of the internal standard tetracosane, added to 1 mL of 10% w/w KOH in methanol and mixed vigorously for 5 min. The upper hexane phase was diluted to match the linearity range of the GC–MS instrument. Then, samples were split-injected (1 μL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) carrying a

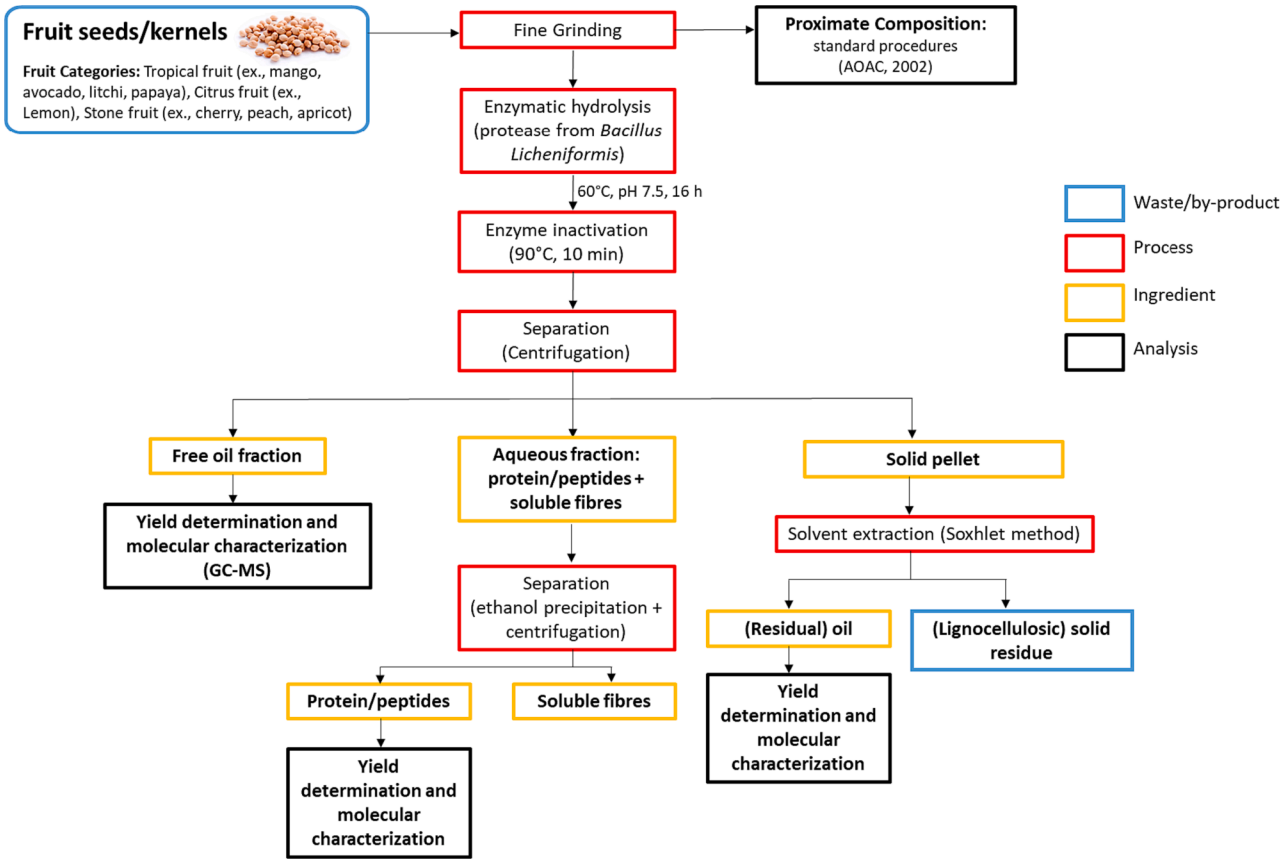

Fig. 1. Workflow of the process performed to extract and isolate oil and protein from fruit seed and kernel by-products.

SUPELCOWAX® 10 capillary column (30 m × 0.25 mm × 0.25 μm, Supelco, Bellafonte, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). Identification and concentration of each detected fatty acid methyl ester (FAME) was determined in relation to the concentration of the internal standard, after calculating the response factors, using the Supelco® 37 Component FAME Mix. Finally, results were expressed as relative percentages of total FAMEs.

2.4.2. Unsaponifiable fraction

The unsaponifiable fraction was extracted and silylated according to ISO 12228-1:2014. Briefly, 5 g of fruit oil and 100 mL of a 2.2 N potassium hydroxide solution in ethanol–water (8:2 v/v) were put into a 250 mL flask; saponification was carried out by boiling and stirring the sample for 1 h. After cooling, 100 mL of distilled water were added, and the sample transferred to a separating funnel and extracted 4 times with 50 mL of diethyl ether. The ether extracts were pooled into a separating funnel and washed with distilled water (50 mL each time), until the wash gave a neutral reaction. The wash water was removed, and the organic sample was dried with anhydrous sodium sulphate, filtered, and taken to dryness. Then, the residue was dissolved in 5 mL of hexane and added to 1 mL of 5-alfa-cholestan-3-beta-ol solution (internal standard, at 0.3 mg/mL in hexane). The unsaponifiable matter was isolated by silica gel (60 Å, 230–400 mesh particle size, 40–63 μm) column chromatography and eluted with a solution of hexane–ethyl acetate (8:2 v/v). Finally, after the solution was filtered and taken to dryness, the residue was sylilated with 600 μL of hexamethyldisilazane (HMDS) and 300 μL of trichloromethylsilane (TCMS) at 60 °C for 1 h and diluted with 1 mL of hexane. The composition of the fractions was determined by split injecting 1 μL of the sylilated solutions in the GC–MS system.

The GC–MS analysis was performed on a 7820A gas chromatograph coupled to an 5977B mass selective detector (Agilent technologies, Santa Clara, CA, USA) with a J&W DB-5 ms (Agilent technologies, Santa Clara, CA, USA) capillary column (temperature: 80 °C for 2 min, 15 °C/min until 280 °C, 280 °C for 20 min). Acquisition mode: scan (*m/z* 40–550). Unsaponifiable components were identified and quantified, by means of the internal standard (5-alfa-cholestan-3-beta-ol), as previously reported (Caligiani, Bonzanini, Palla, Cirlini & Bruni, 2010).

2.5. Molecular characterization of fruit protein hydrolysates

2.5.1 . Amino acids profile by UPLC-ESI/MS

The amino acid profile of fruit protein hydrolysates was investigated according to a previous protocol, slightly adapted (Fuso et al., 2021). For sample preparation, five hundred milligrams of lyophilized sample were hydrolyzed with 6 mL of 6 N HCl at 110 °C for 23 h, then the internal standard (7.5 mL of 5 mM DL-norleucine in water) was added and filtered. The final volume was adjusted at 50 mL with double distilled water and 10 μL of each sample were transferred into a 1.5 mL-tube and subjected to derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), according to the manufacturer instruction. The resulting derivatized samples were diluted with 200 μL of deionized water before injecting in the UPLC system. The UPLC/ESI-MS analysis was performed by using an ACQUITYUPLC® separation system with an Acquity BEH C18 column (1.7 μm, 2.1 × 150 mm, Waters, Milford, MA, USA). The mobile phase was composed of H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 7 min, from 100% A to 75.6% A and 24.4% B by linear gradient from 8 to 28 min, isocratic 100% B from 29 to 32 min, isocratic 100% A from 33 to 45 min. The flow rate was set at 0.25 mL/min, injection volume 2 μL, column temperature 35 °C and sample temperature 18 °C. Detection was performed by using Waters SQ mass spectrometer as previously described (Fuso et al., 2021). The software

used for data processing was MassLynx™ V4.0 (Waters, Milford, MA, USA). Finally, calibration was performed with standard solutions at five concentration levels (1.5, 1, 0.1, 0.05 and 0.01 mM) prepared by mixing 40 μL of DL-norleucine (2.5 mM), different volumes of 2.5 mM Amino Acid Standard H solution and deionized water.

2.5.2. Determination of hydrolysis degree

The protein degree of hydrolysis (% DH), as the integrity of protein fraction after proteolysis, was obtained by using o-phthaldialdehyde (OPA) method previously described (Spellman, McEvoy, O'Cuinn, & Fitsgerald, 2003), slightly adapted. The OPA/NAC (*N*-acetylcysteine) reagent (100 mL) was prepared by combining 10 mL of 50 mM OPA (in methanol) and 10 mL of 50 mM NAC (in methanol), 5 mL of 20% (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminium foil and stirred for 1 h before use. The OPA assay was carried out by adding 20 μL of sample (or standard) to 2.4 mL of OPA/NAC reagent. The absorbance was measured at 340 nm with JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklahoma City, OK, U.S.A) *vs* a control cell containing the reagent and 20 μL of the phosphate buffer solution (10 mM Na_2HPO_4/NaH_2PO_4) used for each sample. The intrinsic absorbance of the sample was measured before OPA addition and subtracted. A standard curve was prepared by using L-isoleucine (at five concentration levels ranging between 0.1 and 2 mg/mL). The % DH was calculated by the following formula:

$$\% \, DH = (A/B) \times 100 \quad (1)$$

where:

A = moles of free nitrogen atoms from alpha amino groups after hydrolysis (determined by OPA assay).

B = total moles of nitrogen atoms in solution before hydrolysis, calculated by the ratio of total grams of proteins and the average of residual amino acids molecular mass (Mw 110).

2.6. Determination of extraction yields and data analysis

The effect of acid hydrolysis on lipid extraction as pre-treatment was evaluated as percentage increase of extracted fat compared to the percentage of extracted fat by using conventional Soxhlet method (by using only organic solvent), according to the following equation:

$$\% \text{ increase} = [(\% \text{ fat}_{\text{acid hydrolisis}} - \% \text{ fat}_{\text{Soxhlet}})/ \% \text{ fat}_{\text{acid hydrolysis}}] * 100 \quad (2)$$

Where:

$\% \text{ fat}_{\text{acid hydrolisis}}$ = % of fat determined after acid hydrolysis + Soxhlet extraction.

$\% \text{ fat}_{\text{Soxhlet}}$ = % of fat determined by conventional Soxhlet extraction (only organic solvent).

After proteolysis, total lipid yield (%) of fruit seed and kernel oil was determined according to the following formula:

$$\textit{Lipid yield (LY, \%)} = [(WSF_g + WFAP_g)/ WFBP_g] * 100 \quad (3)$$

Where:

WSF_g = weight (g) surfaced fat after proteolysis (when present).

$WFAP_g$ = weight (g) extracted fat from the pellet after proteolysis.

$WFBP_g$ = weight (g) extracted fat from fruit seeds/kernels (by acid hydrolysis and Soxhlet extraction) before proteolysis.

Finally, the enzymatic extraction yields of proteins were determined as percentages by comparing the total nitrogen solubilized after the enzymatic hydrolysis (supernatant) and the total nitrogen in the raw seeds/kernels, according to the following equation:

$$\text{Protein yield (\%)} = [N_{\text{after enzymatic hydrolysis}} / N_{\text{raw substrate}}] *100 \quad (4)$$

The contribution of salts containing nitrogen in the extraction systems was considered and subtracted when necessary.

Finally, the data obtained were processed using descriptive statistics to quantitatively describe/summarize features of interest. To this aim, all data are presented as means of independent duplicate or triplicate analysis ± standard deviation (SD). Then, the Student's *t*-test was performed for comparison of conventional and enzymatic-assisted extraction methods (see Table 3). The level of significance (α) was set at 0.05 and p-values < 0.05 were considered as statistically significant. Statistical data elaboration was performed by using Microsoft® Excel® and SPSS software v. 29.0 (Chicago, IL, USA).

3. Results and discussion

3.1. Chemical characterization

3.1.1. Proximate composition

The proximate composition of fruit seeds and kernels, including moisture, protein, lipid, total dietary fibre (TDF), and ash contents, is of great importance for their further exploitation.

The results reported in Table 1 revealed different nutritional profiles based on fruit species.

As previously documented (Fuso et al., 2023; Kumoro, Alhanif & Wardhani, 2020), fruit seeds and kernels are good sources of dietary fibres. Among the analysed fruit biomasses, the kernels of peach, cherry and apricot were found to be the richest in TDF, ranging between 77 ± 2% and 93 ± 2%. Compared to these percentages, the results suggested lower content of TDF in exotic fruits which ranged between 18.1 ± 0.4% (i.e., avocado seeds) and 37 ± 1% (i.e., mango seeds). Besides, other authors (Hussain et al., 2022) suggested that the major nutrients in the exotic fruit seeds and kernels are digestible carbohydrates, in agreement with our results obtained with avocado seeds (73 ± 1% DM). Instead, papaya seeds were richer in proteins (32 ± 1%).

Also, the protein fraction of pumpkin seeds (48 ± 1%) resulted the highest in comparison with other nutrients and with that of other fruit seeds/kernels, in agreement with previous data on other pumpkin varieties (Glew et al., 2006). Lemon seeds resulted quite good as protein source (8.87 ± 0.05%), as already reported (Fuso et al., 2023). The protein percentage of lemon seeds was comparable with those of avocado (7 ± 1%) and litchi (7.1 ± 0.2%) seeds; stone fruit kernels and mango seeds were the lowest in proteins, especially peach kernels with a mean value of 2.95 ± 0.02%.

Different amount of fat was determined based on fruit species. Lemon seeds showed the greatest percentage of fat (37.9 ± 0.9%) in accordance with previous values reported for *Citrus* sp. (Anwar et al.,

Table 1
Proximate composition of the analysed fruit sources (% w/w Dry Weight, DW). Values are means ± standard deviation (SD) of three replicates.

	ash	protein	fat[1]	total dietary fibres (TDF)	others[2]
substrate					
Peach kernels	1.4 ± 0.3	2.95 ± 0.02	10.7 ± 0.1	77 ± 2	8.1 ± 0.2
Cherry kernels	1.48 ± 0.01	4.541 ± 0.003	8.4 ± 0.9	81 ± 2	4.7 ± 0.1
Apricot kernels	1.0 ± 0.2	5.237 ± 0.002	5.3 ± 0.1	93 ± 2	–
Lemon seeds	1.4 ± 0.1	8.87 ± 0.05	37.9 ± 0.9	44 ± 1	7.4 ± 0.1
Mango seeds	1.9 ± 0.1	5.1 ± 0.01	7.2 ± 0.1	37 ± 1	49 ± 1
Papaya seeds	0.08 ± 0.01	32 ± 1	18 ± 1	36 ± 1	13.9 ± 0.3
Avocado seeds	0.039 ± 0.001	7 ± 1	2.2 ± 0.1	18.1 ± 0.4	73 ± 1
Litchi seeds	0.014 ± 0.002	7.1 ± 0.2	3.2 ± 0.1	33 ± 1	57 ± 1
Pumpkin seeds	0.060 ± 0.004	48 ± 1.2	13 ± 1	40 ± 1	–

[1] fat extracted combining acid hydrolysis (acid pre-treatment) and Soxhlet extraction; [2] others mean digestible carbohydrates obtained by difference.

2008), followed by papaya seeds (18 ± 1%) and pumpkin seeds (13 ± 1%). Among stone fruits of *Prunus genus*, peach kernels resulted the highest in fat content with 10.7 ± 0.1%, then cherry and apricot kernels with 8.4 ± 0.9% and 5.3 ± 0.1%, respectively. Regarding other exotic fruits, the mean fat content of mango seeds resulted 7.2 ± 0.1%, whereas litchi and avocado seeds resulted the lowest in fat (3.2 ± 0.1% and 2.2 ± 0.1%, respectively). Besides, in the case of avocado fruit, the oil tends to lower in seeds and accumulate in the mesocarp throughout the fruit developmental period (Ge, Xiangshu, Yuanzheng, Yang & Zhan, 2021). But, except for lemon and avocado seeds, the amount of the extracted lipids was mostly under-estimated than that previously reported for other fruit species, i.e., about 30–40% of fat on dry weight (DW) has been suggested for pumpkin seeds (Hussain et al., 2022) and apricot kernels (Alpaslan & Hayta, 2006), and about 20–30% on DW for papaya seeds (Kumoro et al., 2020) and peach (Pelentir, Block, Rodrigues Monteiro Fritz, & Reginatto, 2011) and cherry kernels (Yilmaz, & Gökmen, 2013).

This is probably due to the scarce extractability of the starting substrate, where the oil fraction mainly remains stored in cellular compartments, namely oleosomes, externally protected by lignocellulosic matter (Konopka, Roszkowska, Czaplicki & Tańska, 2016). As discussed below (paragraph 3.2.1), the acidic hydrolysis performed before the diethyl ether solvent extraction was not always able to totally disrupt the plant cell wall of all these fruit species and to maximize the oil extraction recovery. Indeed, the acid hydrolysis significantly improved the oil extractability depending on the starting substrate, such as peach, cherry, litchi and lemon seeds/kernels (with positive increment percentages in fat extraction >100%), whereas the negative percentage increase obtained for mango and pumpkin seeds suggested their totally resistance to the acidic environment.

3.1.1. Fatty acid profiles

Fruit oil quality/composition of the ether-extracts of acid hydrolysates was determined by fatty acid profile. The data obtained are reported in Table S1.

In agreement with previous studies (Górnaś, Rudzińska, Raczyk, Mišina, Soliven & Segliņa, 2016), fruit seed oils were mostly rich in total monounsaturated fatty acids (MUFA) for high oleic acid (C18:1, generally more than 25% and till 60–70% for peach and papaya seed oils), and polyunsaturated fatty acids (PUFA) for high linoleic acid (C18:2n-6, till about 40% especially in cherry and apricot kernels and lemon and pumpkin seeds) and α-linolenic acid (C18:3n-3, till about 15% in lemon seeds). Among PUFA, about 5% of alpha-eleostearic acid (9-cis,11-trans,13-trans, C18:3n-5) was determined in cherry kernel oil, as previously found (Górnaś et al., 2016). This compound, a conjugated isomer of linolenic acid, is a lipophilic bioactive lipid with antioxidant activity and suggested to influence body fat distribution and lipid metabolism (Yuan, Chen & Li, 2014). Instead, mango seed oil resulted the richest in saturated fatty acids (total SFA, till 60%) with more than 45% of stearic acid (C18:0), showing a cocoa butter- like fatty acid profile (Kumoro et al., 2020). Interestingly, mango seed oil is documented (Jahurul et al., 2014) to be free from toxic compounds like cyanogenic glycosides, thus suggesting its potential applications in the food industry without safety concerns.

As for mango seed oil, the seed oils of the other analysed exotic fruits (papaya, avocado and litchi) resulted richer in total SFA (30–50%) than the other fruit species, due to the higher content of palmitic acid (C16:0) (at about 25–30%), which increased the ratio of SFA/UFA till 1 (especially litchi seed oil).

Unusual fatty acids, such as cyclopropanic fatty acids, were also detected in litchi seed oil as previously suggested (Gaydou, Ralaimanarivo & Bianchini, 1993), mainly dihydrosterculic acid (*cis*-9,10-methyleneoctadecanoic acid) which accounted for about 7% of total fatty acids. Overall, the few amounts of total PUFA (<5%), such as linoleic acid observed in papaya seed oil (only in traces) combined with high level of oleic acid (more than 60%), indicate stronger stability against oxidation (Puangsri, Abdulkarim & Ghazali, 2005). Finally, all fatty acid profiles presented only traces of medium chain fatty acids (C12:0-C14:0).

3.2. Enzymatic hydrolysis of fruit seeds/kernels

In the present study, a one pot protease treatment was carried out on various fruit seeds/kernels. As previously reported (Talekar, Patti, Singh, Vijayraghavan & Arora, 2018), the enzymatic reaction broke down the cytoplasmic matrix of the seeds/kernels releasing the oil and protein hydrolysates in the aqueous medium.

In the following sections, the results in terms of recovery, yields and the molecular characterization of the extracted fractions (i.e., oil and protein) after the proteolysis, including the comparison with acid and organic solvent extractions, will be presented, and discussed in more detail.

3.2.1. Fruit oil extraction and molecular characterization

The lipid yields obtained after organic solvent extractions (with or without acid pre-treatment) and after the proteolysis process are reported in Table 2.

As described above (paragraph 2.2), the crude fat content of fruit seeds/kernels was determined by Soxhlet extraction after acid hydrolysis as a pre-treatment in order to improve the oil extractability of the raw lignocellulosic materials and data accuracy of the proximate composition (reported in Table 1).

However, as reported in Table 2, this method significantly increased the oil quantification only for some substrates, resulting in high positive increment percentages for peach (+727%) and cherry kernels (+492%), lemon (+933%) and litchi seeds (+121%), whereas it quite improved the oil recovery of papaya kernels (+55%) and slightly in avocado seeds (+11%). Then, it did not increase the oil recovery from the other substrates, such as apricot kernels and especially mango and pumpkin seeds, resulting both in a negative percentage increase.

After the enzymatic hydrolysis, about 10–30% of the total fruit oil surfaced from three substrates (i.e., lemon, mango, and pumpkin seeds) and free oils could be recovered for further characterization after centrifugation (as described below). At the end of the enzymatic process, most of the fruit oils remained in the residual pellets (1–5% on fresh weight, FW of seed/kernel residues), which in turn were subjected to organic solvent extraction (as Fig. 1) to calculate the total LY (%) according to Eq. [3] after the proteolysis.

As reported in Table 2, the total average LY (%) obtained from apricot kernels and avocado seeds resulted greater than 100%. This result confirms that the fat content was quite under-estimated by previous proximate analysis, as previously observed (see paragraph 3.1.1), but, at the same time, it suggests that the enzyme reaction improved the oil extractability from the residual pellet compared to the acid hydrolysis.

The results also suggested that both the enzymatic process and the acid pre-treatment before Soxhlet extraction improved the oil recovery from peach kernels and litchi seeds, whereas the oil extractability of cherry kernels and lemon seeds was higher after the acid pre-treatment than the enzyme reaction. The seeds of mango, pumpkin and papaya mostly resulted both acid- and proteolytic resistant as the acid pre-treatment did not improve the oil extractability from these substrates but also the enzymatic process led to quite low oil yields, mainly from papaya (18 ± 1%) and pumpkin seeds (50 ± 16%). So, for these substrates, the process needs to be optimized to increase oil yields and combined to both physical and enzymatic pre-treatments of the raw material, as previously suggested (Konopka et al., 2016).

Then, a further molecular characterization of fruit seed/kernel oils was performed on the oils surfaced after proteolysis (i.e., from mango, lemon and pumpkin seeds) in terms of fatty acid profile and unsaponifiable fraction, followed by a comparison with the nutritional composition of the same seed/kernel oil but extracted by acid and organic

Table 2
Percentages of fat obtained after extraction processes (organic, acid + organic and enzymatic-assisted extractions) and lipid yields after proteolysis of fruit seeds/kernels of various fruit categories. Values are means ± SD of two independent extractions.

sources of seeds/ kernels	% extracted fat by conventional Soxhlet extraction (only solvent)[1]	% extracted fat by acid hydrolysis + Soxhlet extraction[1]	% increase after acid pre-treatment[2]	% fat of the residual pellet[3] (obtained by Soxhlet extraction)[1]	% yield of surfaced fat after enzymatic hydrolysis[4]	total % yield of fat after enzymatic hydrolysis[5]
stone fruits						
peach	0.49 ± 0.01	4.05 ± 0.01	+727	2.952 ± 0.001	–	98 ± 2
cherry	1.300 ± 0.001	7.7 ± 0.9	+492	2.2 ± 0.8	–	64 ± 24
apricot	4.01 ± 0.01	4.01 ± 0.01	0	4.976 ± 0.001	–	113 ± 15
citrus fruits						
lemon	2.4 ± 0.5	24.8 ± 0.9	+933	3.7 ± 0.2	33 ± 1	65.2 ± 0.5
exotic fruits						
mango	4.17 ± 0.04	4.12 ± 0.04	−1	1.5 ± 0.3	22 ± 1	77 ± 12
papaya	10.7 ± 0.1	16.6 ± 0.1	+55	0.57 ± 0.01	–	18 ± 1
avocado	1.8 ± 0.1	2.0 ± 0.1	+11	0.8 ± 0.2	–	117 ± 25
litchi	1.4 ± 0.1	3.1 ± 0.1	+121	0.9 ± 0.1	–	102 ± 16
others						
pumpkin	11.2 ± 0.9	7.9 ± 0.6	−29	1.8 ± 0.7	13 ± 1	50 ± 16

[1] values are calculated on Fresh Weight (FW) of fruit seeds/kernels; [2] values are calculated according to Eq. (2); [3] obtained after enzymatic hydrolysis (see Fig. 1); [4] values are calculated on total fat (%) extracted by acid hydrolysis and Soxhlet method; [5] values are calculated according to Eq. (3).

Table 3
Characterization of fruit oils obtained from lemon, mango and pumpkin seeds with different extraction procedures (AP + Sox, Acid Pre-treatment + Soxhlet extraction; EAE, Enzymatic-Assisted Extraction).

FAMEs (rel %)	Lemon seed oil		Mango seed oil		Pumpkin seed oil	
	AP + Sox	*EAE (surfaced fat)*	*AP + Sox*	*EAE (surfaced fat)*	*AP + Sox*	*EAE (surfaced fat)*
C16:0	(15.5 ± 0.7)*	(17.5 ± 0.1)	(9.1 ± 0.2)*	(8.1 ± 0.1)	(14 ± 1)	(15.6 ± 0.5)
C18:2	(38.1 ± 0.9)	(37.0 ± 0.2)	(8.0 ± 0.7)	(8.4 ± 0.1)	(42 ± 2)	(43.1 ± 0.6)
C18:0	(3.8 ± 0.01)*	(5.10 ± 0.09)	(46.9 ± 4.9)	(44.5 ± 0.1)	(8.2 ± 0.6)	(8.5 ± 0.2)
C18:3 (alpha-ln)	(14.5 ± 0.1)*	(13.7 ± 0.2)	(0.55 ± 0.04)	(0.79 ± 0.04)	(0.88 ± 0.02)	(0.84 ± 0.06)
C18:1 (oleic)	(26.5 ± 0.2)*	(25.6 ± 0.3)	(31 ± 1)*	(35.6 ± 0.6)	(31.7 ± 0.6)	(31.3 ± 0.2)
∑ SFA	(20.5 ± 0.9)*	(23.3 ± 0.3)	(60 ± 3)	(55.3 ± 0.4)	(25 ± 2)	(24.8 ± 0.7)
∑ MUFA	(26.8 ± 0.2)*	(26.0 ± 0.2)	(31 ± 1)*	(35.6 ± 0.6)	(32.1 ± 0.6)	(31.3 ± 0.2)
∑ PUFA	(52.6 ± 0.7)*	(50.72 ± 0.05)	(8.6 ± 0.7)	(9.1 ± 0.2)	(43 ± 2)	(43.9 ± 0.5)
∑ total UFA	(79.5 ± 0.9)*	(76.7 ± 0.3)	(40 ± 3)	(44.7 ± 0.8)	(75 ± 3)	(75.2 ± 0.7)
SFA/UFA ratio	0.3	0.3	1.5	1.2	0.3	0.3
unsaponifiable compounds (mg/kg oil)						
α-tocopherols	(783 ± 78)*	(134 ± 17)	–	(26 ± 1)	(16 ± 7)	(25 ± 10)
ß-tocopherols	–	–	–	–	–	(38 ± 10)
Campesterol	(986 ± 148)*	(352 ± 74)	(500 ± 10)	(423 ± 18)	(33 ± 10)	(33 ± 10)
Stigmasterol	–	(138 ± 20)	(500 ± 10)	(598 ± 33)	(24 ± 8)	(25 ± 10)
ß-sitosterol	(4084 ± 999)*	(2636 ± 782)	(2700 ± 270)	(2043 ± 74)	(1004 ± 226)*	(438 ± 12)
Total 4,4-dimethylsterols	(990 ± 313)*	(83 ± 18)	(200 ± 20)	(480 ± 13)*	(11 ± 6)	–
Squalene	(569 ± 57)	–	–	(259 ± 14)	(890 ± 9)*	(46 ± 11)
Total aliphatic alcohols	(1702 ± 281)*	(305 ± 69)	(200 ± 20)	(438 ± 12)*	(217 ± 62)	(1104 ± 91)*
Total phytochemicals	9115*	3649	(4100)	4247	2196*	1710

Notes: data are expressed as mean of replicates ± SD from independent extractions; FAMEs: Fatty Acid Methyl Esters. *Statistically significant difference between values from two extraction methods for each type of fruit seed oil (Student's *t*-test, $p < 0.05$). SFA = Saturated Fatty Acids; MUFA = Monounsaturated Fatty Acids; PUFA = Polysaturated Fatty Acids.

solvents, to highlight eventual selective extractions of a part of triglycerides and also on the unsaponifiable fraction after the protease reaction. The results obtained agree with literature data (Ryan, Galvin, O'Connor, Maguire & O'Brien, 2007) and are reported in Table 3.

The differences in significance ($p < 0.05$), shown in Table 3, made no significant differences in the fatty acid profiles of fruit seed oils obtained by acid/solvent-extraction and that obtained by the enzyme reaction. Nevertheless, solvent extraction combined to acid pre-treatment determined higher concentrations especially of unsaponifiable compounds (as ß-sitosterol, which resulted the most abundant), compared to the enzyme-assisted extraction. The increased concentration of lipid compounds (both fatty acids and unsaponifiable compounds) is mostly evidenced for lemon seed oil. This is probably related to the increased oil yield previously observed, and it suggests a higher susceptibility of the membranes of the lemon seed oil bodies to acidic environment, which induces an increased solubilization of lipid substances.

Instead, the fatty acid profiles of the oils extracted from the pellet by organic solvent reflected those which were obtained from the starting material with the same extraction protocol (see Table S1).

3.2.2. Yields, hydrolysis degree and amino acid profiles of protein hydrolysates

After the protease reaction, fruit proteins were recovered in the form of protein hydrolysates from the aqueous medium, preceded by 95% ethanol precipitation of the soluble fibres (that causes the co-precipitation of starch, when present in the starting material). As mentioned in the Materials and Methods section (paragraph 2.3), no residual oil was found in the supernatant by hexane-washing. So, the aqueous phase was lyophilized, and extraction yields and percent hydrolysis degree (% DH) were determined. The results are reported in Table 4.

The data reported in Table 4 showed a total average protein yield of 55 ± 11% for all fruit categories. The maximum value (71 ± 2%) was obtained for mango seed, whereas the minimum protein yield (33 ± 4%)

Table 4
Protein yields[1] and percent hydrolysis degree (% DH)[2] after the enzymatic-assisted extraction of different fruit seeds and kernels. Yield values and % DH are expressed as mean ± SD of duplicates of independent extractions.

sources	total yield % after enzymatic hydrolysis	% DH	average peptide length (100/% DH)	average Mw (Da)[3]
stone fruits				
peach	64 ± 1	3 ± 1	33	3630
cherry	70 ± 2	3 ± 1	33	3630
apricot	47 ± 1	3 ± 1	33	3630
citrus fruits				
lemon	50 ± 2	8.8 ± 0.4	11	1210
exotic fruits				
mango	71 ± 2	4 ± 1	25	2750
papaya	60 ± 11	2.5 ± 0.8	40	4400
avocado	54 ± 18	3 ± 1	33	3630
litchi	49 ± 3	3 ± 2	33	3630
others				
pumpkin	33 ± 4	8 ± 2	12	1320

[1] determined according to Eq. (4); [2] calculated according to Eq. (1); [3] calculated considering mean amino acid Mw of 110 Da.

was determined for pumpkin seed.

These results clearly suggested that the protease treatment gave different protein yields, mainly depending on the type of the starting residue source and the different susceptibility to the proteolysis as it occurred for the oil fraction processing. However, the protein extraction yields found in this study are comparable with those reported in the literature based on the use of conventional solvent extractions of fruit seeds and kernels (Karabiber & Yilmaz, 2017).

Also, Table 4 showed the % DH of the resultant protein hydrolysates. The % DH significantly influences the potential allergenicity, the bioactive properties and techno-functional properties of the peptides, which strongly depend on their molecular weight distribution (Udenigwe & Aluko, 2012).

The % DH average found in this study mostly resulted between 3 and 4%, except for that of papaya seed proteins with the lowest value (2.5 ± 0.8%) and those obtained from lemon and pumpkin seed protein hydrolysates, which resulted the highest (almost 10%) among fruit seed varieties. From the % DH, it is possible to determine the average peptide chain length of hydrolysates which can give information not only for the physicochemical and functional properties of the protein hydrolysate, but also for their potential hypo allergenicity. Fruit seed/kernel protein hydrolysates have an average peptide length spanning from 11 to 12 aminoacidic residues (lemon and pumpkin) to much larger peptides (25–40 aminoacidic residues). Depending on the molecular mass distribution of peptide fragments, hypoallergenic formulas are classified as extensively (<3 kDa), or partially (3–10 kDa) hydrolysed. Partially hydrolysed mixtures are designed to prevent potential sensitisation (3–10 kDa), while the extensively hydrolysed ones to avoid clinical symptoms (<3 kDa) (Lozano-Ojalvo & López-Fandiño, 2018). An extensively hydrolysed mixture of protein hydrolysates generally consists of peptides that at 95% have a molecular mass < 3 kDa (Bøgh, Barkholt, & Madsen, 2015). This means that some of the fruit hydrolysates (namely lemon, mango and pumpkin), can be promising for obtaining new potential hypoallergenic products. Obviously, to define a hypoallergenic product, immunological tests are needed to actually prove the lack of allergenicity.

Moreover, the amino acid profile of the fruit protein hydrolysates was evaluated. The data obtained are presented in Table 5 and expressed as mg/g of protein. Aspartic acid + its amidated derivative asparagine, glutamic acid + its derivative form glutamine, and phenylalanine +

Table 5
Total amino acid profile of the protein hydrolysates (after soluble fibre ethanol precipitation) extracted from different fruit seeds/kernels.

	seed/kernel sources																																							
Amino acids (mg/g protein)	**Lemon**			**Cherry**			**Peach**			**Apricot**			**Mango**			**Avocado**			**Papaya**			**Litchi**			**Pumpkin**		
Ala	55	±	2	54.7	±	0.3	93	±	1	70	±	1	106	±	1	103	±	13	36	±	1	73	±	7	44.3	±	0.1
Asn + Asp	88	±	7	158	±	10	231	±	10	179	±	10	243.2	±	0.1	184.5	±	31	157	±	2	128	±	14	82	±	3
Arg	87	±	1	56	±	2	38	±	1	40	±	1	37	±	4	32	±	4	84	±	5	48	±	17	108	±	14
Gly	74.7	±	0.3	78	±	3	66	±	5	69	±	5	52	±	1	47	±	13	33	±	1	43	±	2	43	±	5
Hys	13	±	8	17	±	1	34	±	1	20	±	1	25	±	1	32	±	12	39	±	1	25	±	6	25	±	3
Ile	33	±	1	28	±	3	30	±	1	31	±	1	30	±	1	35	±	1	38	±	1	23	±	1	33	±	3
Leu	74	±	5	52	±	1	45	±	1	54	±	1	33	±	1	61	±	3	60.3	±	0.5	42.0	±	0.2	67	±	8
Met	12	±	1	11	±	2	14	±	1	13	±	1	17	±	2	18	±	4	16	±	1	13.63	±	0.01	21	±	3
Phe + Tyr	100.2	±	0.1	77	±	6	90	±	5	86	±	5	79	±	5	81	±	9	85	±	6	152	±	4	106	±	7
Pro	43.0	±	0.2	32	±	1	31	±	1	38	±	1	24	±	1	35	±	1	45	±	1	71	±	5	41	±	1
Ser	57	±	7	53	±	2	56	±	2	57	±	2	93	±	1	85	±	37	41	±	1	99	±	33	62	±	1
Thr	39.4	±	0.2	33	±	4	31	±	1	36	±	1	33	±	1	71	±	8	49	±	2	16	±	2	65	±	8
Val	52	±	6	38	±	3	46	±	3	45	±	3	47.4	±	0.3	56	±	3	46.0	±	0.6	33	±	2	39	±	5
Lys	9	±	1	10	±	2	12	±	1	10	±	1	11	±	1	40	±	26	31	±	4	56	±	9	62	±	15
Gln + Glu	163	±	20	179	±	9	102	±	10	172	±	10	154	±	1	155	±	37	241	±	7	178	±	26	202	±	12

tyrosine were reported as total sum.

As reported in Table 5, the results suggested that all fruit protein hydrolysates are incomplete protein sources from a nutritional point of view, compared to the FAO/WHO standard reference proteins (FAO/WHO/UNU, 2007). Noticeably, the limiting amino acids are mostly histidine, methionine, and lysine in all fruit seed/kernel protein hydrolysates. Nevertheless, all fruit categories resulted rich in phenylalanine + tyrosine in the range of 77–152 mg/g protein, especially lemon, pumpkin, and litchi seed protein hydrolysates with values higher than 100 mg/g protein, compared to soy protein (78–97 mg/g protein) (Young & Pellett 1991). Also, all fruit hydrolysed proteins contain high amounts of glutamine + glutamic acid (ranging between 102 and 241 mg/g protein) and asparagine + aspartic acid (ranging between 88 and 243 mg/g protein).

In general, the amino acid profiles found in the protein hydrolysates from seeds and kernels of *Prunus* genus and exotic fruit resulted quite similar in terms of amino acid distribution, in agreement with previous reported data (Kumoro et al., 2020).

Among seed protein hydrolysates, that obtained from lemon seed resulted quite rich in branched chain amino acids, such as valine (52 ± 6), leucine (74 ± 5) and isoleucine (33 ± 1), exceeding the standards set by the FAO/WHO for human diet (39, 59, 30 mg/g protein, respectively). Its amino acid profile agrees with that reported for Persian lime seed protein isolate of which leucine, valine, lysine, and phenylalanine were the major essential amino acids (Karabiber & Yilmaz, 2017). Other authors also suggested lemon protein hydrolysates as a favourable protein source for applications in the food industry, due to its functional properties in stabilizing emulsions (Fathollahy, Farmani, Kasaai & Hamishehkar 2021).

The pumpkin seed protein hydrolysate contained low proportions especially of histidine and methionine, but useful amount of arginine, in accordance with previously reported data (Glew et al., 2006). Besides, in addition to their nutritional value, protein hydrolysates have great potential to improve technological and sensory properties of foods (Oreopoulou & Tzia, 2007) and to be used as functional food ingredients, due to their antioxidant (Fathollahy et al., 2021; Karabiber & Yilmaz, 2017), antimicrobial and anti-inflammatory functions (Nan et al., 2007). So, the results obtained in this study encourage further investigation on the functional properties of fruit protein hydrolysates to be exploited in various food products.

4. Conclusion

Waste fruit seeds and kernels are underexploited and interesting substrates for the recovery of alternative functional ingredients for different industrial sectors. So, the present study provided a useful strategy for the exploitation of raw material sources that are still underutilized and generally treated as waste.

The proteolytic process applied on different fruit seeds and kernels was an effective method to obtain from a single step (i) the concomitant and total separation of the lipid fraction with high nutritional value from the aqueous solution and (ii) to recover proteins as protein hydrolysates purified from the soluble fibre fraction to be explored for their potential functional properties in foods.

The behaviour of the lipidic fraction during extraction is strongly dependent on the specific fruit matrix. Most of the fruit seeds needed an acid pre-treatment to fully recover the oil. However, the enzymatic treatment has probably a role in disaggregating the plant cell wall matrix, allowing better oil extraction in a more sustainable way with respect to the use of strong acids.

The enzymatic hydrolysis also represents an effective method to extract and isolate proteins from fruit seeds/kernels, preserving quality and making them more accessible for their future use as feed/food supplements. Protein hydrolysates, as opposed to intact protein, are more rapidly digested and absorbed, and their potential hypo allergenicity is also important. However, due to the lack of some essential amino acids, protein hydrolysates need to be co-formulated with other protein sources and further investigation is needed in order to assess their techno-functionality for future industrial production. Moreover, in the optic of a biorefinery approach, further analysis is required to recover and valorise the protease-derived lignocellulosic biomass of the residual pellet. Finally, detailed safety assessment and the advancement from pilot to demonstration scale are needed.

Funding

This research was financially supported by the Programme "FIL-Quota Incentivante" of University of Parma.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100819.

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