Research Article

Potential of Wastewater Valorization after Wet Extraction of Proteins from Faba Bean and Pea Flours

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Abstract

The present study aimed to characterize wastewater fractions obtained after the wet extraction of proteins from legumes. In addition, the suitability of wastewater fractions for the potential recovery of high value-added compounds was also examined, and consequently, the prevention of the environmental impact of these wastes was explored. Similar to the industrial production of proteins, wet alkaline and acidic extractions of proteins from faba bean and pea flours were performed in two stages of extraction. The different wastewater fractions were characterized by measuring their organic matter content, total solids (TS), total dissolved solids (TDS), electrical conductivity (EC), pH, and turbidity. The value-added compounds from these wastewater fractions were quantified, which included the protein content, carbohydrate content, phenolic content, and antioxidant activity. In addition, the phenolic compounds in these factions were identified and quantified. It was observed that the fractions obtained in the first extraction stage had 60%–90% higher organic matter

content, measured as the chemical oxygen demand (COD), compared to the second fractions, indicating a higher environmental impact of the former in case of disposal. The results obtained for COD, TS, TDS, EC, pH, and turbidity demonstrated that microfiltration reduced only the turbidity (85%), and consequently, a decrease was observed in the particulate matter, while there was a practically negligible reduction in the soluble matter. Wastewater from faba exhibited the highest polyphenol content and antioxidant activity, and was, therefore, considered the most valuable fraction for potential valorization.

1. Introduction

The wastewater from industrial processes involving the use of fruits and vegetables contains organic matter and nutrients in huge amounts, which has raised environmental concerns [1]. It remains a challenge for the food industry to implement wastewater strategies for reducing the environmental impact of the disposal and recovering potential bioactive substances. Wastewater from the agro-food industries contains a variety of high value-added nutrients and bioactive compounds, such as proteins, unsaturated fatty acids, dietary fibers, and polyphenols [2], and nowadays, there is a growing demand for healthy food products containing such functional proteins and bioactive ingredients. However, concern regarding the environmental impact of the production of animal proteins is also increasing these days. As a consequence, the exploration of novel sources of plant-derived proteins is gaining momentum. In this context, the recovery of high value-added compounds from the wastewater discarded from the industries using fruits and vegetables is worth exploring. After the recovery of bioactive compounds from the wastewater, subsequent wastewater technologies for cleaning and recycling could be better applied. The food industry has large water consumption, while the reuse of water has been minimal due to legislation constraints and hygiene concerns [3]. Manzocco et al. [4] reported innovative technological solutions based on either physical or chemical strategies to treat the wastewater released from processing plants.

Legume proteins may be isolated from the flours obtained from milled seeds using a wet process of extraction under alkaline or acidic conditions. In this process, low molecular weight water-soluble components and soluble proteins are extracted from the flour [5], and subsequently, the globular proteins are isolated using a selective precipitation step at the isoelectric point. The isolated globular proteins are then neutralized and dried to achieve a final protein content of approximately 85% [5]. Faba beans (*Vicia faba*) and peas (*Pisum sativum*) are legumes that are attracting increasing attention as sources for obtaining plant-based proteins. The chemical compositions of faba beans and peas may vary with the variety, maturity, and growing conditions of the plants. Faba beans contain 55%–62% carbohydrates, 26%–35% proteins, and 0.7%–2.0% fats [6]. The faba bean proteins comprise approximately 80% globulin storage proteins, vicilin, and legumin [7]. Peas contain 60%–65% carbohydrates, 23%–31% proteins, and 1.5%–2.0% fats [8], and the majority of their protein content comprises globulins (55%–65%) and albumins (18%–25%), which together

account for approximately 80% of total protein content [5]. In addition, faba beans and peas contain small amounts of bioactive compounds, such as vitamins, minerals, and polyphenols [8, 9]. Several previous studies have reported the polyphenol content in faba and pea flours, with the phenolic content and antioxidant activity of faba bean flour being higher than that of pea flour [10].

In large-scale industrial processes producing protein isolates, water is consumed in huge amounts, and consequently, high volumes of wastewater are generated. Therefore, developing innovative technologies to recycle and reuse this wastewater generated in protein isolation processes is of great significance. Such technologies should be able to remove the undesirable physical and chemical components while also controlling and preventing the potential growth of pathogenic and degradation-causing organisms [3]. Recently, Chen et al. [2] described the application of different technologies for the recovery of bioactive substances from the wastewater generated from fruit and vegetable processing industries. Physical methods based on membrane separation technologies have been applied previously for water recycling and recovery of the by-products present in wastewaters [3]. Such treatments allow the safe discharge of wastewater after the potential recovery of high value-added components. Ultrafiltration (UF) and nanofiltration (NF) membranes have been used to recover bioactive compounds from the wastewater generated from the agro-food industry [11]. However, such methods have certain limitations as the waste streams released from different industries are quite different, and the recovery technologies must, therefore, be tailored for each specific condition [2].

The present study was aimed to characterize different wastewater fractions obtained after the alkaline and acidic wet extraction processes applied for the production of protein isolates from faba bean and pea flours, adapting the procedure performed on the industrial upscale to the laboratory scale. Data were obtained depending on the type of extraction (alkaline and acidic fractions), the extraction process (first and second stages), and the type of matrix used (faba bean and pea flours). In addition, the value-added compounds, including carbohydrates and phenolic compounds, that could potentially be recovered from these wastewater fractions were identified and quantified.

2. Materials and Methods

2.1 Chemicals

The chemical reagents used were: hydrochloric acid solution 32 wt. % in H_2O, FCC (Sigma-Aldrich, Merck, Darmstadt, Germany); sodium hydroxide pellets EMPROVE® ESSENTIAL (Merck, Darmstadt, Germany); gallic acid monohydrate ACS reagent ≥98.0% (Sigma-Aldrich, Merck, Darmstadt, Germany); sodium bicarbonate ACS reagent ≥99.7% (Merck, Darmstadt, Germany); methanol ACS EMSURE® (Merck, Darmstadt, Germany); Folin and Ciocalteu's Phenol reagent (MP Biomedicals, Valiant Co. Ltd., China); 2,2-Diphenyl-1-picrylhydrazyl (free radical) 95% (Alfa Aesar, Thermo Fisher Scientific, Kandel, Germany); absolute ethanol ≥99.8%; AnalaR NORMAPUR® ACS (VWR, West Chester, Pennsylvania, United States); formic acid 98% for ACS analysis (PanReac AppliChem, ITW Reagents, Chicago, USA); acetonitrile Optima LC/MS grade (Fisher Chemical, Pittsburgh, PA, USA); and ultrapure water (MilliQ Advantage A10 water purification system, Millipore, Billerica, MA, USA).

2.2 Extraction of Proteins from Faba Bean and Pea Flours and Collection of Wastewater Fractions

Flours from faba beans (*Vicia faba* L.) and peas (*Pisum sativum* L.) provided by local farmers (Västra Götaland, Sweden) were utilized as the plant material for protein extraction. Both alkaline and acidic extraction processes were performed for each plant material based on the method reported by Swanson [12] with a few modifications. The alkaline extraction process was conducted by mixing 25 g of flour with distilled water in a ratio of 1:15 (w/v) and adjusting the pH to 9.5 using 1 M NaOH. The mixture was stirred for 1 h and then centrifuged at 3400 rpm for 15 min. The supernatant was retrieved, and its pH was adjusted to 4.5 using 0.5 M HCl to precipitate the proteins at this isoelectric point. The mixture was then centrifuged at 3400 rpm for 15 min, and the protein isolate was acquired while the supernatant was collected as the first wastewater fraction from the alkaline extraction. Subsequently, a washing step was conducted by mixing the protein isolate into distilled water (1:10 w/v) for 5 min and then centrifuging the mixture at 3400 rpm for 15 min. The collected supernatant was designated as the second wastewater fraction from the alkaline extraction, while the precipitate was retained as the washed protein isolate.

The acidic extraction process was performed by progressively mixing 25 g of flour with acidified (pH 2.5) distilled water (1:15 w/v), and a stable pH of 2.5 was maintained by continuous adjustment with 0.5 M HCl. The mixture was stirred for 1 h and then centrifuged at 3400 rpm for 15 min. The supernatant was retrieved, and its pH was adjusted to the isoelectric point at pH 4.5 using 1 M NaOH, followed by centrifugation at 3400 rpm for 15 min. The protein isolate was acquired, and the supernatant was collected as the first wastewater fraction from the acidic extraction. Subsequently, a washing step was conducted by mixing the protein isolate with distilled water (1:10 w/v) for 5 min and then centrifuging the mixture at 3400 rpm for 15 min. The collected was supernatant designated as the second wastewater fraction from the acidic extraction, while the precipitate was retained as the washed protein isolate.

2.3 Characterization of the Faba Bean and Pea Wastewater Fractions

Eight different wastewater fractions were obtained according to the type of matrix used (faba bean and pea flours), the type of extraction process applied (alkaline and acidic extractions), and the stage of the extraction process (first and second wastewater fractions). These wastewater fractions were characterized for their physicochemical parameters, which included chemical oxygen demand (COD), total solids (TS), total dissolved solids (TDS), electrical conductivity (EC), pH, and turbidity.

Prior to the characterization process, the wastewater fractions were filtrated through two different filters of pore sizes 0.2 μm (Acrodisc®, Pall) and 0.1 μm (Acrodisc®, Pall) to evaluate the effect of membrane filtration. Subsequently, the physicochemical parameters of COD, TS, TDS, EC, pH, and turbidity, were measured using the 0.2-μm filtrates, while only TS, TDS, EC, and pH were measured for the 0.1-μm filtrates. COD was measured using a spectrophotometer (Spectroquant, NOVA 60, Merck) using the standard photometric method [13]. Total solids were determined using the APHA standard method 2540 B [13], and TDS were measured using a digital refractometer (HI 96801, Hanna Instruments). Electrical conductivity and pH were measured using a conductometer and pH meter, respectively (FiveGoTM equipped with LE407 and LE703 IP67 probes, Mettler-Toledo GmbH). Turbidity was measured using a turbidimeter (Turbiquant 300 IR, Merck).

2.4 Analysis of High Value-Added Compounds

Potential high value-added compounds in the wastewater fractions were identified and analyzed. The protein content in all the wastewater fractions was measured as described in a previous study for the analysis of the total protein content [14]. The carbohydrate content was analyzed after filtration through a 0.22-µm filter (16532-K 0.22 µm, polyethersulfone, and high-flow syringe, Sartorius Minisart), and the individual separation and quantification were performed using an HPLC-Refractive Index (HPLC-RI) Detector. The HPLC system (Merck Hitachi, Germany) was equipped with a column thermostat (Thermostat Merck L-5025 Column), an interface (Interface D-7000), an autosampler (AS-2000A Autosampler), a pump (L-6200 Intelligent Pump), and an RI detector (Elite LaChrom VWR Hitachi L-2490), and was controlled using software (D-7000 HSM). The analysis was conducted on a C8 column (Phenomenex Rezex RHM-Monosaccharide, 300 mm × 7.80 mm, 8 microns, 00H-0132-K0). The mobile phase was water in an isocratic gradient, used at a flow rate of 0.5 mL min^{-1}. The injection volume was 20 µL, and the total time of analysis was 25 min.

The phenolic profile was analyzed through HPLC, while the total phenolic content was measured using the Folin-Ciocalteu reagent method [15] with a few modifications. Gallic acid was used as the standard for quantification, and the results were expressed as milligrams of gallic acid equivalents (mg GAeq). Prior to the identification and quantification of individual phenolic compounds in the samples, a concentration step through a solid-phase extraction cartridge (Chromafix C18 ec cartridge S; Macherey-Nagel) was conducted. The wastewater fractions were filtrated through the cartridge (10 mL) prior to being activated using methanol. The polyphenol fraction was retained in the cartridge and later eluted with methanol (0.5 mL). The methanolic fraction was then filtered using a 0.22-µm filter (Millipore Millex–GV, PVDF) and analyzed using HPLC-DAD-MSn/ESI.

The HPLC system was equipped with a diode array detector and a mass detector in series (HPLC 1100 Series, Agilent Technologies, Waldbronn, Germany), a binary pump (Model G1312A), an autosampler (Model G1313A), a degasser (Model G1322A), and a photodiode array detector (G1315B). The system was controlled using software ChemStation (Agilent, v.08.03). The mass detector was an ion-trap spectrometer (Model G2445A) equipped with an ESI interface that was controlled using software LCMSD (Agilent, v. 4.1). Mass spectrometry data were acquired in the negative ionization mode under the ionization conditions of 350 °C temperature and 4 kV voltage. Nitrogen was used as the nebulizer gas with a pressure of 65 psi and a flow rate of 11 L·min^{-1}. The full scan covered the mass range of m/z 50 to m/z 1200. Collision-induced fragmentation was performed using nitrogen as the collision gas and a voltage of 1V. Total ion chromatograms were recorded as full-scan mass spectra (MS), with the MS2 for the fragmentation of the three major significant fragment ions derived from the [M-H] and MS3 breakdown of the significant fragment ions derived from the MS2. The analysis was conducted on a C18 HPLC column (Agilent Pursuit XRs 100 Å, 250 mm × 4 mm, 5 µm particle size). The mobile phase consisted of two solvents – a mixture of water and formic acid in a ratio of 99:1 (v:v) (A) and acetonitrile (B). The gradient began with 3% B and reached 9% in a linear gradient at 5 min, 16% at 15 min, 50% at 50 min, and 90% at 52 min, and finally returning to the initial conditions at 57 min. The total time of analysis was 62 min, the flow rate was 0.8 mL·min^{-1}, and the injection volume was 20 µL. The chromatograms were recorded at 280 nm, 320 nm, 340 nm, 360 nm, and 520 nm. The phenolic standards used for identification were weighed on a microanalytical balance (0.001 mg) and then dissolved in methanol. Two groups

of standards were prepared: 1) quercetin, caffeic acid, ferulic acid, and hydroxybenzoic acid, and 2) rutin and p-coumaric acid.

The antioxidant activity of the samples was analyzed using a previously reported protocol [16] with a few modifications, and the effective concentration (EC50) was calculated [17].

2.5 Statistical Analysis

All data were analyzed using PASW Statistics 25 for Windows (SPSS Inc., Chicago, IL, USA). Student t-test (independent two-sample t-test), with the significance threshold of $p < 0.05$, was conducted for comparing the means in pairs. Student t-test was applied to study the variations in the parameters evaluated. The data were compared according to the stage of the extraction process (first and second wastewater fractions), the type of extraction (alkaline and acidic extraction fractions), and the type of matrix used (faba bean and pea flours). The filtered and unfiltered samples were grouped together for the statistical analysis (COD, TS, TDS, EC, and pH parameters) as the differences between them were not quantitatively relevant.

3. Results and Discussion

3.1 Characterization of the Wastewater Fractions from Faba Bean and Pea Protein Extraction Processes

In the case of the wastewater fractions acquired from faba bean, most of the evaluated parameters differed significantly ($p < 0.001$) between the first and second fractions (Table 1), with the COD, TS, TDS, and EC values approximately 66%–89%, 88%–92%, 89%–90%, and 87%–90% higher, respectively, in the first fraction compared to the second one. In regard to the type of extraction, certain significant differences were observed, although these differences were not quantitatively relevant, except for the COD parameter measured in the second fraction ($p < 0.001$), which was approximately 59% higher in the acidic extraction than in the alkaline one.

Table 1 Physicochemical parameters of the wastewater fractions from the alkaline and acidic protein extractions from faba bean.

Parameters	Extraction type	Wastewater fractions	
		First	Second
COD (mg·L^{-1})	Alkaline	5940 ±165	653 ±17 ***
	Acidic	4746 ±37	1599 ±79 ***
		+++	+++
Total solids (g·L^{-1})	Alkaline	10.2 ±0.1	0.8 ±0.1 ***
	Acidic	9.4 ±0.8	1.1 ±0.2 ***
		+	++
Total dissolved solids (°Brix)	Alkaline	0.9 ±0.1	0.1 ±0.1***
	Acidic	1.0 ±0.1	0.1 ±0.0***

		ns	ns
Electrical conductivity (mS·cm^{-1})	Alkaline	4.02 ±0.06	0.40 ±0.02 ***
	Acidic	4.49 ±0.40	0.58 ±0.03 ***
		ns	++
pH	Alkaline	4.53 ±0.01	4.64 ±0.04 **
	Acidic	4.46 ±0.01	4.50 ±0.08 ns
		+++	+

Values are expressed as the mean ±standard deviation of at least three replicates, corresponding to the data of all samples evaluated for each condition (unfiltered and filtered). The significant differences based on the extraction stage are presented in the same row, and those for the type of extraction are presented in the same column. Statistical analysis results according to Student t-test are denoted as + ($p < 0.05$), ++ ($p < 0.01$), +++ ($p < 0.001$), and ns (not significant).

In the case of wastewater fractions from pea flour, most of the evaluated parameters differed significantly ($p < 0.001$) between the first and second fractions (Table 2), with the COD, TS, TDS, and EC values approximately 86%–91%, 89%–91%, 88%–90%, and 88%–90% higher, respectively, in the first fraction compared to the second one. In regard to the type of extraction, certain significant differences were observed, although these differences were not quantitatively relevant, except for the COD parameter measured in the second wastewater fraction ($p < 0.001$), which was higher [by approximately 36%] in the acidic extraction fraction. When the impact of the matrix was compared (faba bean and pea), significant differences were observed, although quantitative differences were obtained only for the COD parameter measured in the first extraction stage ($p < 0.001$), which was approximately 25%–39% higher in the pea fraction than in the faba fraction, regardless of the type of extraction process used.

Table 2 Physicochemical parameters of the wastewater fractions from the alkaline and acidic protein extractions from pea.

Parameters	Extraction type	Wastewater fractions	
		First	**Second**
COD (mg·L^{-1})	Alkaline	7909 ±127	700 ±37 ***
	Acidic	7794 ±332	1095 ±45 ***
		ns	+++
Total solids (g·L^{-1})	Alkaline	9.2 ±0.3	0.8 ±0.0 ***
	Acidic	9.8 ±0.4	1.1 ±0.0 ***
		+	+++
Total dissolved solids (°Brix)	Alkaline	0.8 ±0.1	0.1 ±0.0 ***
	Acidic	1.0 ±0.1	0.1 ±0.1 ***

		+	ns
Electrical conductivity (mS·cm^{-1})	Alkaline	2.57 ±0.08	0.30 ±0.00 ***
	Acidic	4.13 ±0.20	0.42 ±0.03 ***
		+++	++
pH	Alkaline	4.58 ±0.02	4.63 ±0.04 ns
	Acidic	4.42 ±0.02	4.17 ±0.04 **
		++	+++

Values are expressed as the mean ±standard deviation of at least three replicates, corresponding to the data of all samples evaluated for each condition (unfiltered and filtered). The significant differences for the extraction stage are presented in the same row, and those for the type of extraction are presented in the same column. The statistical analysis results according to Student t-test are denoted as + ($p < 0.05$), ++ ($p < 0.01$), +++ ($p < 0.001$), and ns (not significant).

Recently, Chen et al. [2] indicated that the wastewaters from the food industry contained a high organic load, particularly the wastewater from the industries producing soybean products, which had high levels of COD (16000 mg·L^{-1}), TS (18200 mg·L^{-1}), and total suspended solids (TSS) (4000 mg·L^{-1}). In the present study, the analysis of the first wastewater fractions revealed high COD and TS as the soluble inorganic, and organic matter present in the flour remained in the supernatant after the separation of the protein isolate. The second fraction exhibited a lower COD and TS, which was the result of the washing performed after the first extraction.

In addition, the results revealed that certain factors, such as the pH of the process, the type of extraction, and the matrix used, caused slight differences in the COD values. Therefore, all the fractions collected, regardless of the extraction stage, the type of extraction, and the matrix used, contained high loads of COD, which indicated a negative environmental impact in case of disposal and warranted the use of a wastewater recovery strategy.

Turbidity results also demonstrated significant differences between the wastewater fractions depending on the extraction stage and the type of extraction ($p < 0.001$ in most cases). Turbidity in the first extraction stage was approximately 90%–97% higher than that in the second stage, particularly in the alkaline extraction. In the acidic extraction, turbidity was approximately 60%–90% higher in the second extraction stage compared to the first one. As observed with the other evaluated parameters as well, turbidity results also indicated that the pH of the first extraction step of protein isolation was a critical influencing factor.

Wastewater treatment technologies, such as membrane filtration, ozonation, adsorption, and solvent extraction, might reduce the organic matter present in the wastewater fractions [2], thereby clearing the fractions and reducing the environmental impact in case of disposal. In the present study, membrane filtration was used as a first step in the treatment strategy to remove organic matter from the wastewater fractions, which resulted in a significant decrease ($p < 0.001$) in turbidity (Table 3). Reduction in turbidity has been previously associated with the decline of particulate COD [18]. Although this fraction obtained after filtration might also contain relevant products to be valorized, this analysis was not performed in the present study. The results demonstrated that filtration reduced approximately 85%–99% of turbidity, regardless of the type of

extraction and the matrix used, which indicated that the reduction of particulate matter or total suspended solids was achieved after the filtration process and the dissolved solutes were recuperated in the effluent. Van Haute et al. [18] also observed a great decrease in turbidity along with a slight decrease in COD, indicating the negligible contribution of particulate matter to the total COD. According to the COD and TS values, it was inferred that the organic matter present in the wastewater fractions was constituted of dissolved solutes. Microfiltration (MF) (0.1–1.0 μm pore size) has been applied previously to remove suspended or particulate solids and organic colloids [19]. In the present study, MF or ultrafiltration (UF) (0.01–0.1 μm pore size) resulted in a clear reduction in particulate matter or total suspended solids in all the filtered wastewater fractions, while the dissolved constituents were not removed. In contrast, membrane technologies such as nanofiltration (NF) and reverse osmosis (RO), designed for the removal of dissolved chemical constituents, could be suitable for this purpose [20–22]. The rejection rate of NF is approximately 40%–60% of TDS in contrast to the value of 90%–98% achieved in RO [20–23]. These technologies, therefore, could be effective in series, i.e., using MF or UF as a pretreatment procedure. Aerobic and anaerobic digestions or flocculation, which have been previously applied in the treatment of wastewater from fruit and vegetable processing plants [2], could also be used for the wastewater fractions in the present study.

Table 3 Turbidity (NTU) of the wastewater fractions from the alkaline and acidic protein extractions from faba bean and pea flours and their corresponding filtrates.

Matrix	Extraction type	Wastewater fractions and filtrates			
		First	First filtrate	Second	Second filtrate
Faba bean	Alkaline	183.9 ±0.3	1.7 ±0.1 ***	5.6 ±0.4	0.2 ±0.0 **
	Acidic	9.2 ±0.2 +++	1.3 ±0.1 ***	73.5 ±4.9 ++	3.3 ±0.0 **
Pea	Alkaline	130.6 ±0.3	4.4 ±0.3 ***	10.1 ±0.5	0.1 ±0.0 **
	Acidic	12.7 ±0.3 +++	1.7 ±0.1 ***	39.7 ±0.0 ++	0.8 ±0.0 ***

Values are expressed as the mean ± standard deviation of 2 replicates. The significant differences for the same extraction stage and filtration are presented in the same row, and those for the type of extraction are presented in the same column. The statistical analysis results according to Student t-test are denoted as + (p < 0.05), ++ (p < 0.01), +++ (p < 0.001), and ns (not significant).

3.2 Potential Recovery of Proteins from the Wastewater Fractions from Faba Bean and Pea

The efficiency of the protein extraction was measured indirectly by determining the total protein content in the wastewater fractions. The wet protein extraction process began with solubilizing the

legume flour in an aqueous medium and adjusting the pH of the solution to either acidic or alkaline range far from the isoelectric point. Subsequently, the solubilized proteins were precipitated at the pH corresponding to their isoelectric point [24]. The impacts of the extraction stage, the type of extraction, and the matrix used were studied. Significant differences were observed when the fractions were compared based on the extraction stages (Figure 1). Protein content in the first wastewater fractions was approximately 65%–88% higher (p < 0.01 in most cases), regardless of the matrix used and the extraction type. These results demonstrated that a portion of protein did not precipitate at the isoelectric point while another was solubilized during the washing step after removing the protein isolate. In general, the protein content in the wastewater fractions indicated that after the extraction step, a portion of the soluble proteins remained in the supernatant (first wastewater fraction) even after the isoelectric precipitation. The protein loss might be related to the type of proteins, i.e., a few proteins might be soluble in the acidic or alkaline media, while having slightly different isoelectric points compared to the ones selected in the present study. Moreover, the presence of protein remaining in the supernatant even after precipitation could be attributed to the fact that certain proteins might require a longer time or a higher acid/alkali molarity for precipitation.
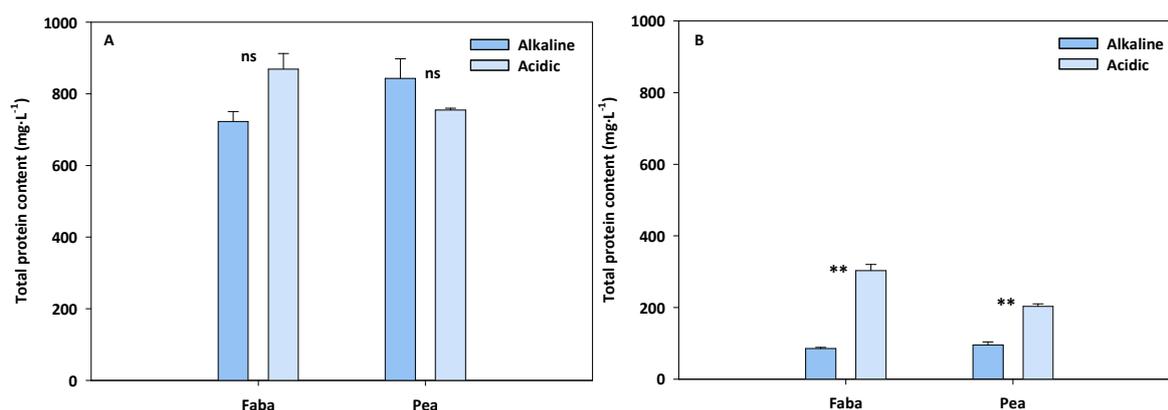

Figure 1 Total protein content in (A) the first wastewater fractions from the alkaline and acidic protein extractions from faba bean and pea; (B) the second wastewater fractions from the alkaline and acidic protein extractions from faba bean and pea. The significant differences for the type of extraction (alkaline/acidic) and in each extraction stage according to Student t-test are denoted as * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and ns (not significant).

When the type of extraction was compared in the statistical analysis, no differences were revealed in the first wastewater fractions, while significant differences were observed in the second wastewater fractions (p < 0.01). The protein content in the wastewater fractions from the acidic extraction from faba bean and pea was 72% and 53% higher, respectively, compared to the corresponding alkaline extraction fractions. The difference in the protein content between the acidic and alkaline treatment wastewater fractions could be attributed to the fact that the acid-solubilized proteins are closer to the isoelectric point compared to those solubilized under alkaline conditions.

In regard to protein isolation efficiency, extraction using acidic media is reportedly a less favorable process due to lower cell wall degradation caused by the acid, which may lead to low protein diffusion to the acidic medium. Moreover, the applied acid pH is closer to the isoelectric point of the protein compared to the pH in the alkaline experiments, which results in lower protein solubility [25]. In general, alkaline extraction generates higher extraction yields compared to acidic extractions. Nevertheless, acidic extraction may be preferable in certain cases due to the different techno-functional properties of the extracts, besides preventing extreme pH conditions that may cause protein denaturation and high consumption of acids, alkalis, and water [26]. The protein content in the different isolates varied with the extraction pH and the legume type. Under both acidic and alkaline extraction conditions, the isolates from peas (pH 2.5, 99.5% DW; pH 9.0, 93% DW) exhibited a higher protein content compared to the faba bean isolates (pH 2.5, 88.9% DW; pH 9.0, 82.3% DW). In addition, both faba bean and pea isolates exhibited higher protein purity under acidic extraction.

Various technologies, such as foam separation or UF, have been applied to recover soybean protein from wastewaters [2]. A system based on two-stage foam separation technology reportedly achieved recoveries of approximately 80%, while a combination of UF and RO has also been used for recovering soluble substances [2, 27, 28]. These technologies could be effective in the recovery of the remnant proteins present in the wastewater fractions.

3.3 Potential Recovery of Carbohydrates from the Wastewater Fractions from Faba Bean and Peas

The HPLC-RI analysis of the wastewater fractions revealed the presence of carbohydrates (Table 4). Among the four sugars investigated, namely, sucrose, fructose, glucose, and galactose, only sucrose was identified and quantified, and the rest were either not present or were at concentrations below the detection limit. The extraction stage, the type of extraction, and the impact of the matrix used were statistically analyzed. The analysis for sucrose revealed a higher content of approximately 82%–92% in the first wastewater fractions compared to the second ones ($p < 0.001$), regardless of the type of extraction and the matrix used. In addition, the sucrose content after the first acidic extraction from pea was higher (67%) than that in the faba bean wastewater from acidic extraction ($p < 0.001$), while the reverse was observed under alkaline extraction. Kontos et al. [29] studied the methodologies for the recovery and purification of phenolic compounds from the carbohydrates blend in olive mill wastewater and observed that NF membranes were useful for separating both types of compounds, retaining approximately 95% of sucrose from tyrosol. The authors recommended applying a sequence of membrane technologies, such as vacuum distillation, freeze, drying, and melt crystallization, to recover value-added compounds from olive mill wastewater.

Table 4 Sucrose content ($mg \cdot L^{-1}$) in the wastewater fractions from the alkaline and acidic protein extractions from faba bean and pea.

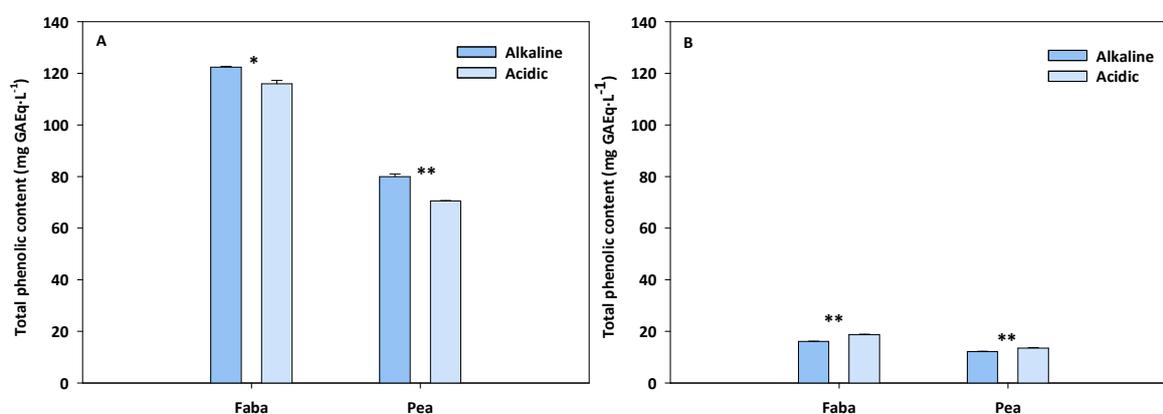| Matrix	Extraction type	Wastewater fractions	
		First	Second
Faba	Alkaline	1598 ±29	115 ±5 ***
	Acidic	726 ±21	125 ±7 ***

		+++	ns
Pea	Alkaline	1215 ±10	138 ±13 ***
	Acidic	2168 ±147	189 ±31 ***
		+++	ns

Values are expressed as the mean ±standard deviation of 3 replicates. The significant differences for the extraction stage are presented in the same row, and those for the type of extraction are presented in the same column. The statistical analysis results according to Student t-test are denoted as + ($p < 0.05$), ++ ($p < 0.01$), +++ ($p < 0.001$), and ns (not significant).

3.4 Potential Recovery of Phenolic Compounds from the Wastewater Fractions from Faba Bean and Peas

The analysis of the total phenolic content in the wastewater fractions was conducted, and the statistical analysis was performed concerning the extraction stage, the type of extraction, and the impact of the matrix used. The total phenolic content was approximately 81%–87% higher in the first wastewater fractions ($p < 0.001$) compared to the second one, regardless of the extraction type and matrix used (Figure 2). When the first wastewater fractions were compared, the phenolic content in the wastewater fraction from faba bean was approximately 34%–39% higher ($p < 0.001$) than that in the pea fraction, regardless of the type of extraction.

Figure 2 Total phenolic content in (A) the first wastewater fractions from the alkaline and acidic protein extractions from faba bean and pea; (B) the second wastewater fraction from the alkaline and acidic protein extractions from faba bean and pea. The significant differences for the type of extraction (alkaline/acidic) and in each extraction stage according to Student t-test are denoted as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and ns (not significant).

The identification and quantification of individual phenolic compounds were focused on the first wastewater fractions as these had the highest total phenolic content. The HPLC-DAD-MSn analysis of the wastewater fractions from faba bean and pea revealed differences in the individual phenolic compounds. The wastewater fractions from the alkaline and acidic extractions from faba bean had similar chromatograms with close signals and low-intensity peaks, rendering the identification

difficult (Figure S1). Two main flavonoid glucosides identified were quercetin-rhamnoside-glucoside and kaempferol-rhamnoside-glucoside (Table 5). The content of kaempferol glucoside was double the quercetin content in both alkaline and acidic wastewater fractions. In pea wastewater fractions, the chromatograms for alkaline and acidic extractions were similar, with the peaks for more intense compared to those obtained for faba beans (Figure S2). Caffeic acid-glucoside, trans-feruloyl-malic acid, trans ferulic acid, trans-p-coumaroyl-malic acid, and p-hydroxy benzoyl-malic acid glucoside were identified and quantified using the corresponding standards (Table 6). In addition, the flavonoid kaempferol-triglucoside was identified and quantified as rutin. In pea, the content of phenolic compounds was slightly higher in the acidic fraction than in the alkaline fraction.

Table 5 Characterization and quantification of phenolic compounds in the first wastewater fractions from the alkaline and acidic protein extractions from faba bean.

	Group	Compound	Rt (min)	λmax (nm)	[M-H]⁻	MS² fragments	MS³ fragments	Quantified indirectly as	Concentration (mg·L⁻¹)
Alkaline extraction	Flavonoids	Quercetin-rhamnoside-glucoside	21.8	258, 356	609	447	301	Quercetin	0.15
		Kaempferol-rhamnoside-glucoside	23.9	234, 266, 326	593	447, 285		Rutin (Quercetin 3-O-rutinoside)	0.30
Acidic extraction	Flavonoids	Quercetin-rhamnoside-glucoside	21.9	258, 356	609	447	301	Quercetin	0.14
		Kaempferol-rhamnoside-glucoside	23.9	234, 266, 326	593	447, 285		Rutin (Quercetin 3-O-rutinoside)	0.33

Table 6 Characterization and quantification of phenolic compounds in the first wastewater fractions from the alkaline and acidic protein extractions from pea.

	Group	Compound	Rt (min)	λmax (nm)	[M-H]⁻	MS² fragments	MS³ fragments	Quantified indirectly as	Concentration (mg·L⁻¹)
Alkaline extraction	Flavonoids	Kaempferol-triglucoside	22.9	264, 346	771	609	285	Rutin (Quercetin 3-O-rutinoside)	0.92
	Phenolic acids	Caffeic acid-glucoside	14.6	314	341	179	135	Caffeic acid	0.23

		trans feruloyl-malic acid	19.2	240, 320	308	193	134	Ferulic acid	0.91
		trans ferulic acid	24.8	238, 324	193	149	134	Ferulic acid	0.62
		trans *p*-coumaroyl-malic acid	16.9	236, 308	278	163	119	*p*-Coumaric acid	0.64
		p-hydroxybenzoyl-malic acid glucoside	13.5	248	414	252	136	Hydroxybenzoic acid	0.57
Acidic extraction	Flavonoids	Kaempferol-triglucoside	22.9	264, 346	771	609	285	Rutin (Quercetin 3-O-rutinoside)	1.25
	Phenolic acids	Caffeic acid-glucoside	14.6	314	341	179	135	Caffeic acid	0.35
		trans feruloyl-malic acid	19.3	240, 320	308	193	134	Ferulic acid	1.09
		trans ferulic acid	24.9	238, 324	193	149	134	Ferulic acid	0.45
		trans *p*-coumaroyl-malic acid	17.0	236, 308	278	163	119	*p*-Coumaric acid	0.75
		p-hydroxybenzoyl-malic acid glucoside	13.5	248	414	252	136	Hydroxybenzoic acid	0.79

Polyphenols are well recognized for their antioxidant properties, as well as for their contribution to the prevention of various human illnesses (cardiovascular disease, cancer, osteoporosis, diabetes, and neurodegenerative diseases) [11]. Therefore, the recovery of these value-added compounds (polyphenols) from wastewater as by-products recovered from inexpensive input materials is important. In addition, this would be beneficial for wastewater recycling. Several methodologies have been applied to recover polyphenols from the wastewater fractions from fruit and vegetable processing industries. Membrane methodologies, such as NF with different molecular weights cut-offs, have been applied to recover flavonoids, including anthocyanins, from press liquors of orange peel, generating yields ranging from 70% to 89% [30]. Membrane technologies based on RO have reportedly been applied to recover isoflavones from whole soy milk [2, 31]. Moreover, foam fractionation followed by acidic hydrolysis and adsorption on chitosan microspheres has been used for separating isoflavone aglycone from soy whey wastewater [32]. Soybean isoflavones have also been obtained using wastewater protein extraction. However, most of these wastewater fractions are usually disposed of in sewage treatment plants [2, 33]

3.5 Antioxidant Activity of the Wastewater Fractions from Faba Bean and Peas

As in the analysis of phenolic compounds, the antioxidant activity was also determined only in the first wastewater fractions as these fractions had higher polyphenol content. The extraction process and the matrix used were considered for statistical analysis, although significant differences were observed only for the type of matrix used. The effective concentration (EC50) in the pea wastewater fractions was approximately 84%–91%, which was higher than that in the faba bean fractions, regardless of the type of extraction (Figure 3). A lower EC50 value represents a higher antioxidant activity. Therefore, the faba bean wastewater fractions exhibited higher antioxidant activity than the pea fractions.
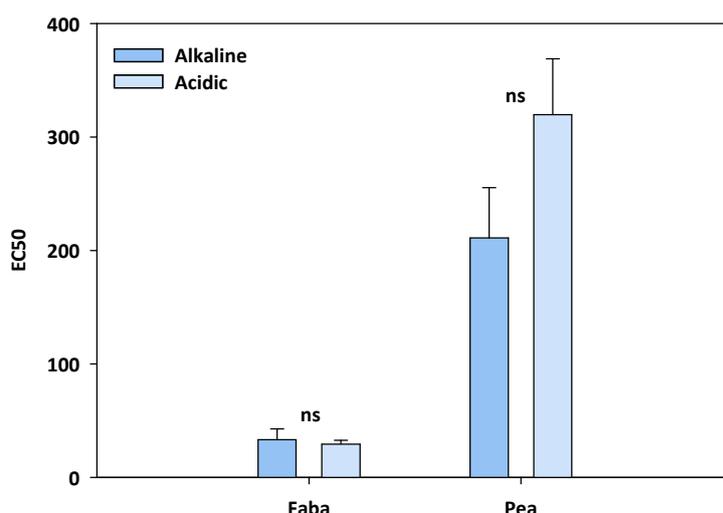

Figure 3 Antioxidant activity in the first wastewater fractions from the alkaline and acidic protein extractions from faba bean and pea. The significant differences for the type of extraction (alkaline/acidic) according to Student t-test are denoted as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and ns (not significant).

While the total phenolic content was 34%–39% higher in the faba bean wastewater than in the pea fraction, the comparison of the antioxidant activities of the two matrices revealed higher differences, which could be associated with the presence of different polyphenol families in pea and faba bean wastewater fractions, as the total antioxidant activity is based on the synergistic effects of the polyphenols present, the metabolite type, and metabolite concentration [34].

4. Conclusions

Huge quantities of wastewater are generated in the industrial processes for the production of protein isolates, which are harmful to the environment. In the present study, all the wastewater fractions from faba bean and peas, particularly the first extraction stage fractions, were indicated to have a negative environmental impact owing to their high organic matter content, reflected as COD and total solids. It is suggested that membrane technologies focused on retaining the dissolved constituents should be implemented, in addition to microfiltration, to reduce the organic and inorganic loads originating from industrial food residues in the wastewater. Prior to implementing the cleaning and recycling strategies, the potential high value-added compounds, such as proteins, sucrose, and polyphenols, should be recovered from the first wastewater fractions, either by alkaline or acidic extraction. The first wastewater fraction from faba bean in the present study exhibited the highest total phenolic content and antioxidant activity, and was, therefore, considered most suitable for the potential valorization. Furthermore, approaches involving the recovery technologies specifically suitable for each group of value-added compounds, such as membrane technologies combined with physicochemical technologies, could also be implemented.

Acknowledgments

Additional Materials

The following additional materials are uploaded on the page corresponding to the present paper.
1. Figure S1: HPLC-DAD chromatogram at 360 nm for the first wastewater fraction from the alkaline (A) and acidic (B) protein extractions from faba bean.
2. Figure S2: HPLC-DAD chromatogram at 320 nm for the first wastewater fraction from the alkaline (A) and acidic (B) protein extractions from pea.

Author Contributions

Sofía Albolafio: Formal analysis, Data curation, writing - original draft, Maria I Gil: Supervision, Writing -review & editing, Ana Allende: Supervision, Epameinondas Xanthakis: Supervision, Writing -review.
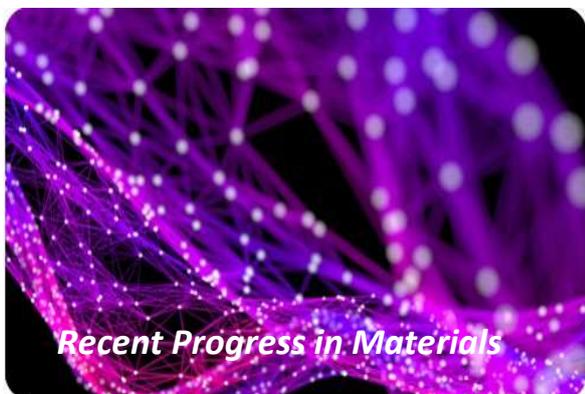
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Competing Interests

The authors have declared that no competing interests exist.

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