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Optimisation of the methodology for obtaining enzymatic protein hydrolysates from an industrial grape seed meal residue

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ABSTRACT

The grape pomace industry produces large quantities of protein-rich seeds, which can be a sustainable non-animal protein source; their techno-functional properties could be exploited to improve the colour stabilisation and modulating the astringency of red wines in warm climates. This study aims to optimise the methodology of obtaining protein hydrolysates from defatted grape seed meal residue. Five assays using different quantities of enzyme and raw materials were considered. Based on the protein purity, hydrolysates yield, colour and molecular weight distribution achieved, optimal conditions were the hydrolysis of the alkaline protein concentrate with the highest amount of enzyme. The products obtained showed the lowest colour parameters, with the lightness contributing the most to the colour differences, which were visually perceptible ($\Delta E^*_{ab} > 3$). The hydrophobic amino acids remained within the peptide sequence, leaving polar and charged amino acids in terminal positions, which could affect the wine equilibrium related to colour stabilisation.

1. Introduction

The implementation of the Plan Nacional de Adaptación al Cambio Climático (PNACC) 2014–2020, which is an important mainstay of European and international policies, is one of the Spain's priority objectives concerning global warming. For viticulture and oenology, the gap between the technological and phenolic maturities in warm climate regions owing to high temperatures and mean annual sun exposure is increasingly evident as climate change progresses (Mira de Orduña, 2010). Among other factors, this time lag contributes to the seeds being unripe at harvest, which leads to a low quantities of phenols and copigments obtained from the seeds (Boulton, 2001). As a result, the obtained wines present low quantitative and qualitative astringency, and it makes difficult to achieve the colour stability during wine ageing or storage in bottles or barrels (Cejudo-Bastante, Rivero-Granados, & Heredia, 2017; Gordillo et al., 2014). In the quest for developing methods that would stabilise the colour of wines, researchers have extensively studied the addition of natural phenolic oenological-derived by-products, such as grape pomace, American non-toasted oak cooperage shavings, and overripe grape seeds (Baca-Bocanegra, Nogales-Bueno, Hernández-Hierro, & Heredia, 2018; Gordillo et al., 2014; Rivero, Gordillo, Jara-Palacios, González-Miret, & Heredia, 2017; Rivero, Jara-Palacios, Gordillo, Heredia, & González-Miret, 2019). This oenological strategy allows mitigating the effects of the deficient phenolic maturity on the quality of the red wines, while enabling the circular production of the oenological by-products.

Grape seeds are a natural source of proteins, which could be of great interest for oenology due to their great techno-functional value for modulating the quality features of red wines, such as appearance, colour, and stability (Gazzola, Vincenzi, Gastaldon, Tolin, Pasini, & Curioni, 2014; Gazzola, Vincenzi, Marangon, Pasini, & Curioni, 2017; Gordillo, Chamizo-González, González-Miret, & Heredia, 2021a; Zhou, Zhang, Liu, & Zhao, 2011; Cejudo-Bastante, Rodríguez-Morgado, Jara-Palacios, Rivas-Gonzalo, Parrado, & Heredia, 2016). The use of the grape seed protein fraction as a biopolymer could be a new sustainable winemaking alternative to the industrial utilisation of animal-derived

Abbreviations: DGSM, defatted grape seed meal; *SEC*, size-exclusion chromatography; BPC, basic protein concentrate; APC, acidic protein concentrate; NPP, non-protein precipitate; SPH, soluble protein hydrolysate; IPH, insoluble protein hydrolysate; MW, molecular weight; ANOVA, analysis of variance; SAA, Sulphur containing amino acids; AAA, aromatic amino acids; HAA, hydrophobic amino acids; TAA, Total amino acids.

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protein (e.g., gelatine, albumin) stabilising agents, and could avert the current legal restrictions because it is a grape-derived product. Thus, the addition of low-molecular-weight (low-MW) peptides derived from grape seeds to wine during the initial stages of the winemaking process could lead to the early stabilisation of the phenolic structure and colour of wines. Moreover, high-MW peptides could be added to wines during the advanced stages of winemaking to achieve later finning and stabilisation, and to prevent precipitation (Ozdal, Capanoglu, & Altay, 2013; Gazzola et al., 2017).

Enzymatic hydrolysis is an eco-friendly and solvent-free alternative to the chemical hydrolysis for extracting and solubilising proteins in aqueous media (Bautista, Hernandez-Pinzon, Alaiz, Parrado, & Millan, 1996; Parrado, Miramontes, Jover, Gutierrez, Collantes de Terán, & Bautista, 2006). The changes in the MWs of peptides and amino acids affect the properties of hydrolysates, enhance their biological and technological functionalities (Chi, Wang, Wang, Zhang, & Deng, 2015), and confer them high stability, good dispersion capacity, and very high solubility (Parrado et al., 2006), which are attributes necessary for the incorporation of peptide concentrates into wines. To accomplish this, it is necessary to dispose of peptide concentrates but, however, no studies about the optimisation of the enzymatic hydrolysis of grape seeds have been performed to date.

Therefore, as part of a larger investigation aimed to modulate the unbalanced astringency and to stabilise the colour of wine using oenological protein sources, the goal of this study was to optimise the methodology for obtaining peptide fractions via enzymatic hydrolysis. The raw material was an industrial defatted grape seed meal (DGSM), residue from the extraction of grape seed oil, which is normally discarded, used for animal feed, or as industrial biomass. We focused on four parameters, viz. the hydrolysates yield, the protein content, the MW distribution obtained via size-exclusion chromatography (*SEC*) of the obtained enzymatic protein hydrolysates, and the colorimetric characteristics of the treated wines using differential tristimulus colorimetry. The amino acid composition and peptide sequence of the optimal products were also scrutinized. To the best of our knowledge, no studies have been previously performed on the effects of the processing and enzymatic conditions on these parameters using this low-cost protein source residue. Since different sources of proteins could present distinct behaviour during the hydrolysis, even using the same enzyme (Tavano, 2013), five assays with different parameters, such as the type of feedstock to be hydrolysed and the extraction conditions, were developed to optimise the methodology of obtaining enzymatic protein hydrolysates.

2. Materials and methods

2.1. Chemicals

HPLC grade water was obtained using a Milli-Q Plus (Millipore Corp., Bedford, MA, USA) water purification system. Na_2HPO_4 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The standards used to analyse the column performance were aprotinin (Sigma-Aldrich, St. Louis, MO, USA), cytochrome *C* (Panreac, Barcelona, Spain), vitamin B_{12} and triglycine (Alfa Aesar, MA, USA), and glycine (Thermo Fisher Scientific, MA, USA). The endoprotease Alcalase® (subtilisin A) (Novozymes®, Bagsvaerd, Denmark) was used to obtain the enzymatic protein hydrolysates, whose enzymatic activity was 2400 U/μL, using azocasein as substrate.

2.2. Samples

The DGSM was supplied by Alvinesa Natural Ingredients S.A. (Daimiel, Ciudad Real, Spain) and consisted of defatted grape seeds mainly from Airén white grapes. Briefly, at the Alvinesa facilities, grape pomace is received as by-product from the winemaking process, and it is subjected to different processes. Concretely, grape pomace was washed extensively with water, destemmed, and dried. Then, the grape seeds were separated from the grape pomace, dried, ground and pelletized for further oil extraction using hexane in an industrial extractor. The residual defatted grape seed meal was desolventised to remove the residual hexane, constituting the industrial DGSM. Afterwards, in the laboratory, the DGSM was ground and passed through a 0.5 mm sieve to obtain a more pulverised raw material, which was subsequently kept in sealed plastic containers and stored in dark at room temperature until further analyses.

2.3. Methodology development

To optimise the methodology of obtaining hydrolysed protein fractions from DGSM, five assays (A1–A5) were conducted. Enzymatic hydrolysis was performed using three starting materials: DGSM, a basic protein concentrate (BPC) of the DGSM, and an acidic protein concentrate (APC) of the DGSM. In addition, two enzyme concentrations (0.3% and 0.6% (v/v)) were tested. A scheme of the assays is shown in Fig. 1. All tests were performed in triplicate in a temperature and pH-controlled bioreactor.

2.3.1. Preparation of the BPC of the DGSM (assays A1 and A2)

The method of Ghribi et al. (2015a) with some modifications was used to prepare the BPC. First, 70 g of DGSM was mixed with 350 mL of distilled water (20:80, (w/v)) and the pH was adjusted to 10.5 with ammonia to facilitate protein solubilisation. Subsequently, the mixture was mechanically stirred at 500 rpm for 3 h at 25 °C and the pH was maintained at 10.5. Afterward, the resulting mixture was centrifuged at 9500 rpm and 4 °C for 15 min. The non-protein precipitate (NPP) was discarded, and the supernatants were pooled and concentrated under vacuum for 2 h until elimination of ammonia.

2.3.2. Preparation of the APC of the DGSM (assay A3)

Once the BPC supernatant was collected as previously described, the pH was lowered to 3.5 with HCl 37% (to avoid using large amount), centrifuged at 9500 rpm and 4 °C for 15 min, separating the supernatant (APCS) and the precipitate (APC). Finally, distilled water was added to APC (1:200 (w/v)) to redissolve the precipitate for the further hydrolysis.

2.3.3. DGSM solution preparation (assays A4 and A5)

Distilled water (350 mL) was added to 70 g of DGSM (20:80, (w/v)), and the pH was adjusted to 8.5, optimal hydrolysis pH of Alcalase enzyme (Ugolini et al., 2015).

2.3.4. Enzymatic hydrolysis (assays A1–A5)

Enzymatic hydrolysis using Alcalase as hydrolytic agent was performed under agitation and alkaline conditions (Parrado, Bautista, Romero, García-Martínez, Friaza, & Tejada, 2008). All hydrolyses were performed at the same conditions of pH, time, and temperature. First, the solutions containing BPC, APC and DGSM were heated at 80 °C for 5 min to inactivate the endogenous enzymes (Ghribi et al., 2015a). Subsequently, the temperature and pH of the mixtures were adjusted to the optimal values for the Alcalase enzyme (50 °C and 8.5, respectively) using the pH-stat method. Afterward, depending on the assay, 0.3 or 0.6% v/v of enzyme was added to the sample and the reaction was conducted at 50 °C and pH 8.5. After 4 h, the enzyme was inactivated by increasing the temperature to 80 °C for 10 min. Later, the pH of the solution was adjusted to 3.5 with HCl 37%. Subsequently, the solution was centrifuged at 9500 rpm for 15 min to separate the soluble and insoluble protein hydrolysates (SPH and IPH, respectively). Both samples were concentrated under vacuum in a rotatory evaporator at 60 °C and lyophilised for further analyses.

2.4. Hydrolysates yield and protein content

The hydrolysates yield ((protein weight of the hydrolysate/protein

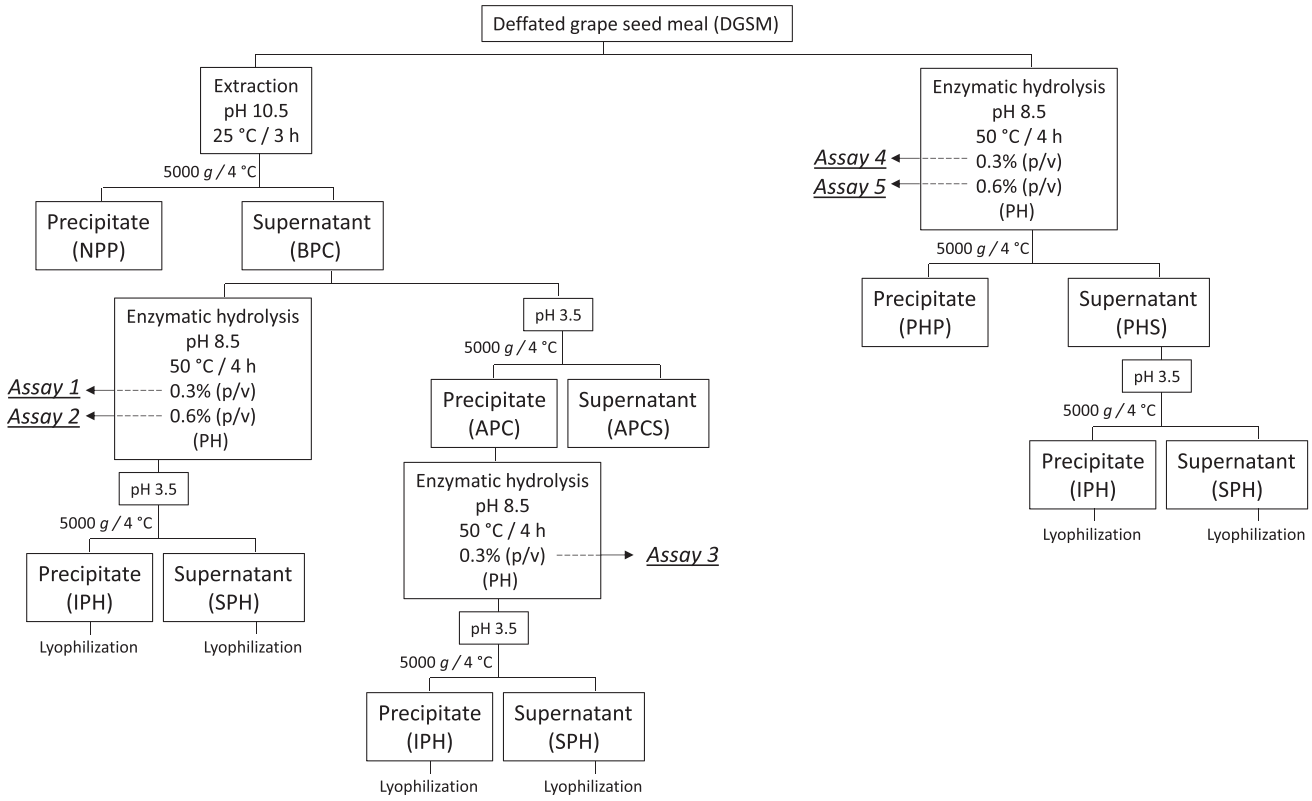

Fig. 1. Schemes of the assays for obtaining protein hydrolysates from the defatted grape seed meal residue.

weight of DGSM) × 100) were calculated. For each sample, the crude protein content was estimated based on the nitrogen content using the standard Kjeldahl method (AOAC, 1995). The protein content was determined by multiplying the total nitrogen content by 5.75. All analyses were carried out in triplicate.

2.5. Molecular weight analysis by size-exclusion chromatography (SEC)

The MW distribution was determined using *SEC*, according to the method of Bautista et al. (1996). Prior to chromatographic analysis, the samples were filtered through a 0.45 μm filter. A Superdex™ 30 Increase 10/300 GL column (optimum separation range of 0.1–7 kDa) was used on an Agilent 1100 chromatography system equipped with a quaternary pump, an automatic injector, an UV–vis diode-array detector, and the ChemStation software (Agilent Technologies, Palo Alto, CA, USA). For the peptide fractions separation, a flow rate of 0.5 mL/min of Na_2HPO_4 50 mM (pH 7.5) in isocratic mode and 25 °C was used. Following the manufacturer's instructions, a mixture of standards was used to cover the range of 100–7000 Da, as follows: cytochrome *C*, 12 500 Da; aprotinin, 6500 Da; vitamin B_{12}, 1355 Da; triglycine, 189 Da; and glycine, 75 Da. The peptide fractions were monitored at 215 and 280 nm.

2.6. Colorimetric analysis

A CM-5® spectrophotometer (Konica Minolta, Tokyo, Japan) was used to perform the colour measurements of the lyophilised products (powder) by diffuse reflectance. Data were collected over the entire visible spectrum (380–770 nm). The CIELAB colorimetric parameters (L^*, a^*, b^*, C^*_{ab}, and h_{ab}) were obtained from the reflectance spectra following the recommendations of the Commission Internationale de L'Eclairage (CIE, 2004), using the CIE 1964 10° Standard Observer and the Standard Illuminant D65, which corresponds to natural daylight. The Euclidean distance between two points in the three-dimensional (L^*, a^*, b^*) CIELAB space were used to calculate colour differences (ΔE^*_{ab}) as follows:

$$\Delta E^*_{ab} = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\right]^{1/2}$$

2.7. Amino acid analysis

The amino acid (AA) composition was obtained by ion-exchange chromatography, and ninhydrin was used for the post-column derivatization. For each sample, 6 mg were hydrolysed with 2 mL of 6 N HCl in sealed glass tubes for 24 h at 110 °C and the pH was adjusted to 2.0 with 10 N, 5 N, and 1 N NaOH. 400 μL of the adjusted samples and 100 μL of a norleucine solution (50 μM as an internal standard) were mixed and filtered through a 0.45-μm nylon filter before being analysed by HPLC. The HPLC system (Biochrom 30+Amino Acid Analyzer HPLC system Ltd., Cambridge, UK) was equipped with a high-pressure PEEK cation exchange column packed with Ultropac 8 cation exchange resin and a UV–vis detector with two wavelengths, 440 and 570 nm for proline and other amino acids, respectively. Quantification was done in triplicate by comparing the amino acid content of the sample with the standard. The amino acids contents were expressed as mg/g protein product.

2.8. Peptide identification by LC–MS/MS

Optimum samples (SPH and IPH of assay A2) were desalted and concentrated onto OMIX Pipette tips C_{18} (Agilent Technologies, Palo Alto, CA, USA). The desalted protein digest was dried, resuspended in 10 mL of 0.1% formic acid and analysed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). The peptides were concentrated by reverse phase chromatography using a 0.1 mm × 20 mm C_{18} RP precolumn (Thermo Scientific), and then separated using a 0.075 mm × 250 mm C_{18} RP column (Thermo Scientific) operating at 0.3 μL/min. Peptides were eluted using a 180-min dual gradient. The gradient profile was set as follows: 5–25% solvent B for 135 min, 25–40% solvent B for

45 min, 40–100% solvent B for 2 min and 100% solvent B for 18 min (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid, 80% acetonitrile in water). ESI ionization was done using a Nano-bore emitters Stainless Steel ID 30 μm (Proxeon) interface at 2.1 kV spray voltage with S-Lens of 60%. The Orbitrap resolution was set at 30.000. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by twenty data dependent MS/MS scans (Top 20), using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35%, and dynamic exclusion applied for 60-seconds periods. Charge-state screening was enabled to reject unassigned and singly charged protonated ions.

2.9. Statistical analysis

Statistical analysis was performed using the Statistica v.8.0 (StatSoft Inc., Tulsa, OK) software. Univariate analysis of variance (ANOVA) was performed using a general linear model program to establish whether the mean scores of the sample data differed significantly from each other. Furthermore, principal component analysis (PCA) was performed to highlight the main contributors to the variance among samples. Along the text, the term significant means p values less than 0.05 ($p < 0.05$) for the statistical differences.

3. Results and discussion

3.1. Obtaining the enzymatic SPH and IPH from DGSM

In this study, we optimised the methodology to obtain different low-MW fractions using a protease. Two fine brownish lyophilised products were obtained from the enzymatic hydrolysis, the soluble supernatant and insoluble precipitate (SPH and IPH, respectively).

The hydrolysates of SPHs and IPHs ranged from 3.8% to 32.4% and 0.5% to 7.1%, respectively (Table 1). Predictably, the protein purities of SPHs and IPHs were significantly higher than that of DGSM (a water-insoluble solid by-product that mainly consists of fibre and proteins, with virtually no lipids), which was in agreement with the results reported by Tejada et al. (2013). The protein purity of DGSM was 10.5%, and it was 60–80% and 40–60% of SPHs and IPHs, respectively. These results were in agreement with the data reported by Feyzi, Varidi, Zare, & Varidi (2015) who noted that alkaline conditions increased the protein purity. Moreover, the protein content of SPHs was similar to those of other raw materials protein hydrolysates, such as cereals (Kaewjumpol, Oruna-Concha, Niranjan, & Thawornchinsombut, 2018), or sunflower seed meal (Ugolini et al., 2015). However, the literature on grape seeds is very scarce.

As expected, the data on the MW distribution of the fractions indicated that the percentage of low-MW peptides or newly formed amino acids in SPHs was higher than that in IPHs. This was ascribed to the Alcalase that breaks down the protein during the enzymatic hydrolysis. Consequently, IPHs consisted mostly of compounds with MW larger than 5 kDa (approximately 65%), whereas the SPHs were rich in low-MW (<5 kDa) peptides and amino acids (70–85%), which is in accordance with the study of Wang, Wei, Li, Bian, & Zhao (2009). Anyway, the MW distribution of IPHs and SPHs greatly depended on the hydrolysed products (Table 1).

Regarding colour, all hydrolysates were located in the first quadrant of the (a^*b^*) plane (positive both a^* and b^* values) (Fig. S1). Hydrolysis led to a wide range of h_{ab} and L^* values, being 36°–63° and 13–56 CIELAB units for the SPHs and 17°–53° and 2–24 CIELAB units for the IPHs, respectively (Table 1).

For a more detailed analysis of each method and starting material used, the effects of hydrolysing BPC or APC compared with that of hydrolysing DGSM, and the effect of the enzyme quantity have been individually discussed using ANOVA analysis (Table 2).

3.2. Effect of hydrolysing the BPC

The effect of hydrolysing BPC was compared with that of hydrolysing DGSM. For that purpose, the SPHs and IPHs obtained using assays A1 and A2 were compared with those obtained using assays A4 and A5.

SPH. The hydrolysates yield of the BPC and DGSM hydrolysis were not significantly ($p > 0.05$) different, unlike their protein purities (Table 1). Specifically, the percentage of proteins obtained via the hydrolysis of BPC (assays A1 and A2) were significantly ($p < 0.05$) higher than those obtained via the DGSM hydrolysis (assays A4 and A5) (Table 2). This was ascribed to the removal of other components (such as fibres) that could difficult to obtain highly pure protein material. In fact, similar protein contents (68%) were reported by Ugolini et al. (2015), who simultaneously extracted and hydrolysed sunflower seed meal. Also it is attributable to the higher alkaline conditions undergoing protein extraction, which increase the negative charge of proteins and the electrostatic repulsion between them; this facilitates the protein solubility due to the protein-water interactions (Lestari, Mulder, & Sanders, 2010; Lv, Jia, Li, Yang, & Zhao, 2011; Baca-Bocanegra, Nogales-Bueno, Hernández-Hierro, & Heredia, 2018). In fact, those authors reported the highest protein extraction at pH 10 compared to other basic pHs lower than 10, sitting this value as the optimum for obtaining a protein concentrate from grape seeds.

The percentages of MW distribution were similar for all samples. Considering that the protein purity positively increased after hydrolysing BPC without altering the percentages of MW distribution, it could be stated that a protein concentration is required prior to enzymatic hydrolysis.

The L^*, C^*_{ab}, and h_{ab} values of the SPHs obtained from assays A1 and A2 were significantly lower than those of the SPHs obtained from assays A4 and A5 (Table 1). Therefore, the SPHs from A1 and A2 were browner and darker than the SPHs obtained using A4 and A5 but chromatically less intense. This behaviour could be related to the CIELAB parameters of the starting raw materials, because BPC was significantly browner and darker than DGSM. Wasswa, Tang, Gu, & Yuan (2007) affirmed that

Table 1
Hydrolysates yield (%), protein content (%), molecular weight (MW) distribution (%) and CIELAB parameters (L^*, C^*_{ab}, h_{ab}) of SPHs and IPHs of the different assays.

		Hydrolysates yield	Protein content	Molecular size fractions			CIELAB parameters		
				MW > 5 kDa	5 > MW > 1 kDa	MW < 1 kDa	L^*	C^*_{ab}	h_{ab}
A1	SPH	32.22 ± 6.70	81.50 ± 1.45	16.7 ± 1.6	38.3 ± 0.8	45.0 ± 2.3	13.67 ± 10.44	14.66 ± 2.50	35.81 ± 16.79
	IPH	6.52 ± 1.75	44.35 ± 3.42	63.9 ± 2.0	19.3 ± 1.3	16.8 ± 0.8	2.45 ± 2.25	11.86 ± 10.48	17.63 ± 3.64
A2	SPH	26.44 ± 2.93	76.01 ± 4.83	14.3 ± 1.6	37.4 ± 0.8	48.3 ± 1.4	18.96 ± 6.32	14.71 ± 4.10	57.52 ± 4.86
	IPH	7.05 ± 0.93	45.27 ± 2.09	64.2 ± 1.4	18.4 ± 1.0	17.4 ± 0.4	2.51 ± 1.38	13.72 ± 5.13	21.33 ± 4.83
A3	SPH	3.86 ± 0.32	65.88 ± 2.46	28.4 ± 4.9	39.0 ± 3.3	32.6 ± 1.6	43.89 ± 7.32	22.07 ± 0.37	59.49 ± 0.74
	IPH	0.56 ± 0.09	58.39 ± 4.88	63.7 ± 9.9	17.0 ± 3.9	19.3 ± 6.1	23.63 ± 6.56	6.05 ± 0.08	52.72 ± 3.19
A4	SPH	32.39 ± 1.50	63.09 ± 1.90	19.5 ± 0.4	35.9 ± 1.1	44.7 ± 1.2	56.18 ± 3.38	28.06 ± 0.38	62.90 ± 1.12
	IPH	3.52 ± 0.51	45.76 ± 1.42	64.3 ± 1.2	20.7 ± 0.6	15.0 ± 0.8	2.29 ± 1.12	13.09 ± 5.52	17.06 ± 1.72
A5	SPH	27.79 ± 8.82	63.45 ± 0.47	15.8 ± 1.2	38.6 ± 1.2	45.6 ± 1.0	56.58 ± 0.62	28.54 ± 1.16	63.39 ± 0.43
	IPH	3.71 ± 0.40	39.96 ± 5.41	63.9 ± 1.0	20.5 ± 0.8	15.6 ± 0.2	5.37 ± 4.91	15.64 ± 12.45	26.58 ± 12.49

SPH, soluble protein hydrolysate. IPH, insoluble protein hydrolysate.

Table 2
ANOVA analysis applied to all the studied parameters for SPHs and IPHs.

	Protein concentrate [b]		Acidification prior to enzymatic hydrolysis [c]		Quantity of enzyme [d]	
	F	*p*	*F*	*p*	*F*	*p*
SPH						
Hydrolysates yield (%)	0.0494	0.82855	***59.3597***	***0.00003*** [a]	2.9993	0.113973
Protein content (%)	***39.986***	***0.00009*** [a]	***89.6500***	***0.00069*** [a]	0.2241	0.64608
MW > 5 kDa (%)	3.2562	0.10131	***15.8989***	***0.01630*** [a]	9.5900	0.01131
5 > MW > 1 kDa (%)	0.5710	0.46734	0.1010	0.76694	1.2230	0.29467
MW < 1 kDa (%)	1.8740	0.20102	***60.7040***	***0.00146*** [a]	4.5530	0.05864
L^*	***132.5794***	***0.00000*** [a]	***16.8627***	***0.01478*** [a]	0.0470	0.83269
a^*	2.8911	0.11991	0.0619	0.81572	1.0576	0.32799
b^*	***122.1512***	***0.000001*** [a]	***45.6364***	***0.00250*** [a]	0.2623	0.61965
C^*_{ab}	***112.450***	***0.000001*** [a]	***25.7507***	***0.00711*** [a]	0.0035	0.95407
h_{ab}	***6.1607***	***0.03243*** [a]	5.9529	0.07122	2.0924	0.17864
IPH						
Hydrolysates yield (%)	***33.1018***	***0.00018*** [a]	***17.1589***	***0.00115*** [a]	0.0969	0.76198
Protein content (%)	0.7750	0.39928	***16.6675***	***0.01507*** [a]	1.2780	0.28465
MW > 5 kDa (%)	0.0000	0.95982	0.0018	0.96799	0.0000	0.99195
5 > MW > 1 kDa (%)	***10.5560***	***0.00874*** [a]	0.9299	0.38949	0.5840	0.46243
MW < 1 kDa (%)	***24.6540***	***0.00076*** [a]	0.5219	0.50999	0.8120	0.38863
L^*	0.68954	0.42570	***27.9872***	***0.00613*** [a]	0.9582	0.35074
a^*	0.01714	0.89844	1.8040	0.25038	0.0233	0.88166
b^*	0.47940	0.50445	0.0553	0.82558	1.3315	0.27537
C^*_{ab}	0.1133	0.74338	0.9227	0.39116	0.2239	0.64623
h_{ab}	0.3015	0.59501	***157.6925***	***0.00023*** [a]	3.0232	0.11272

[a] Values are significant at $p < 0.05$.
[b] A1, A2 and A3.
[c] A3.
[d] A1, A3, A4 *vs* A2, A5.

enzymatic browning reactions could occur during a long-time hydrolysis, which resulted in the decrease of the lightness values of the hydrolysate products. Calculation of colour differences (ΔE^*_{ab}) allowed assessing visual differences between the soluble supernatants of assays A1 and A2, and A4 and A5. The hydrolysis of the BPC (assays A1 and A2) provoked a remarkable effect on colour differences compared to the hydrolysis of DGSM (assays A4 and A5). ΔE^*_{ab} was much higher than 3 CIELAB units, which is considered a threshold value to perceive visual colour differences between samples (Martínez, Melgosa, Pérez, Hita, & Negueruela, 2001). The role of each colour attribute with respect to the total colour was also calculated (Gordillo et al., 2021a; Gordillo, Rivero, Jara-Palacios, González-Miret, & Heredia, 2021b). With this purpose, the quadratic differences in lightness, chroma and hue, with respect to the quadratic total colour difference were calculated ($\Delta^2 L^*/\Delta^2 E^*_{ab}$, $\Delta^2 C^*_{ab}/\Delta^2 E^*_{ab}$, $\Delta^2 H^*_{ab}/\Delta^2 E^*_{ab}$) being high (0.893), small (0.103), and negligible (0.042), respectively. These results indicated that the preparation of protein concentrates mainly affected the quantitative attributes of colour.

IPH. According to the ANOVA results (Table 2), when a protein concentrate was obtained prior to the enzymatic hydrolysis, the protein purity did not change significantly. However, in doing so, a significant higher hydrolysates yield was obtained (assays A1 and A2; Table 1), evidenced that A1 and A2 assays were hydrolysed to a lesser extent compared to A4 and A5.

The obtained fraction with MW larger than >5 kDa) using assays A1 and A2 did not significantly differ from that obtained using assays A4 and A5. However, the percentage of fractions with MW < 1 kDa, and 5 > MW > 1 kDa were significantly higher and lower in assays A1 and A2 compared to assays A4 and A5, respectively. Villanueva et al. (1999) indicated that this could be explained by the Alcalase starts the hydrolysis in the high-MW proteins and new protein bands of low- and medium-MW peptides are formed. As a result, the percentage of with the higher MW bands (>5 kDa) can remain invariable even though the MWs are changed. The IPHs showed similar highly dark brown colorimetric characteristics whether hydrolysis was performed on a protein concentrate or not. Moreover, the ΔE^*_{ab} values of the IPHs were negligible, being not significant differences according to the ANOVA results ($p >$ 0.05).

3.3. Effect of acid precipitation prior to enzymatic hydrolysis

The acid precipitation method has some disadvantages, such as the high consumption of alkaline and acid solutions (because the pH was increased and decreased twice during the process) and the more time-consuming method because of the need to lyophilisation of the product obtained from the first acidification (APC) (Fig. 1). The lyophilisation of APC must be carried out to determine the exact amount of solid, to adjust the quantity of water to be added for hydrolysis, and to fix the solid/water ratio. Conversely, it has been reported that acid conditions facilitate the removal of carbohydrates and other non-protein compounds (Feyzi et al., 2015; 2018). Besides, Baca-Bocanegra et al. (2018) selected the pH 3.5 as the optimum to separate grape seed proteins from the supernatant through isoelectric precipitation in the light of the more quantity of protein obtained in the precipitate. Considering that a decrease in pH could lead to remarkable protein purity, the effect of acidifying the basic supernatant derived from the BPC prior to enzymatic hydrolysis was analysed using the SPHs and IPHs obtained from assays A1 and A3.

SPH. The acidification of the alkaline supernatant (BPC) and hydrolysis of the resulting precipitate (APC) did not achieve the purported significant increase in the percentage of proteins of SPH. In fact, the percentage of proteins of SPH using assay 3 was significantly lower than that of SPH obtained using assay A1 (Table 1). The hydrolysates yield presented similar behaviour, and that of SPH using assay A3 was significantly lower than that of the SPH obtained using assay A1. This could be due to discarding different fractions prior to hydrolysis (NPP and APCS), and therefore alkaline conditions resulted in higher hydrolysates yields than acidic conditions (Feyzi et al., 2018).

The MW distribution was also significantly affected by the starting material, which indicates the low formation of low-MW peptides from APC after hydrolysis. The SPH obtained using assay A3 was significantly lighter and more brownish than that obtained using assay A1, and these two SPHs presented with visually appreciable colour differences (ΔE^*_{ab} > 3), mainly due to their differences in lightness ($\Delta^2 L^*$ = 84.6%),

similarly to the results found by Feyzi et al. (2018).

IPH. The hydrolysates yield of assay A3 was much lower than that of assay A1 without acidification, which was significantly different (Table 1). This could be attributed to consider only a fraction of BPC, because only the target of the hydrolysis reaction (the APC fraction) was considered. This could be the reason why the protein purity of the IPH from assay A3 was significantly higher than that of the IPH from assay A1 (Table 2). Conversely, the MW distributions of these samples were not significantly affected by the starting material, which indicates that the enzyme activity was independent of the preliminary acidification. The different methodologies significantly affected the chromatic characteristics of the IPHs, mainly the L^* and h_{ab} values. Acidification led to a visually perceived ($\Delta E^*_{ab} > 3$) more yellowish product, with a high contribution to lightness ($\Delta^2 L^*$ 88.6%), probably due to the same reason as in its homologous SPH.

3.4. Effect of enzyme quantity

The effect of the quantity of enzyme used for hydrolysis was evaluated by comparing the SPHs and IPHs obtained using assays A1 and A2, and A4 and A5.

SPH. The amount of enzyme did not significantly affect the hydrolysates yield (Table 1), which indicates that higher amounts of Alcalase enzyme did not generate larger amounts of SPH. Similarly, this factor did not significantly affect either the protein percentage or the MW distribution. Given the small differences in the low-MW (<1 kDa) peptides fractions and amino acids among samples, we concluded that higher amounts of enzyme did not increase the hydrolysis degree (Ugolini et al., 2015). This fact could be due to the lack of further accessible cleavages sites in the substrate, according to the findings of Ghribi et al. (2015a) in chickpea protein isolate. Therefore, the enzyme seems to have its own specific hydrolysis profile, which does not change with the amount of enzyme. Moreover, the CIELAB parameters were similar and not significantly different regardless the quantity of enzyme.

IPH. As with SPH samples, no significant ($p < 0.05$) differences were noticed in any of the studied parameters when different enzyme quantities were used on IPH samples (Table 1).

To determine the main parameters that contributed to the differences between samples, a non-supervised pattern recognition statistical analysis (principal component analysis, PCA) was performed (Fig. 2). Nine main significant principal components (PCs) were identified using the Kaiser's criterion (eigenvalues >1), which explained 100% of the total

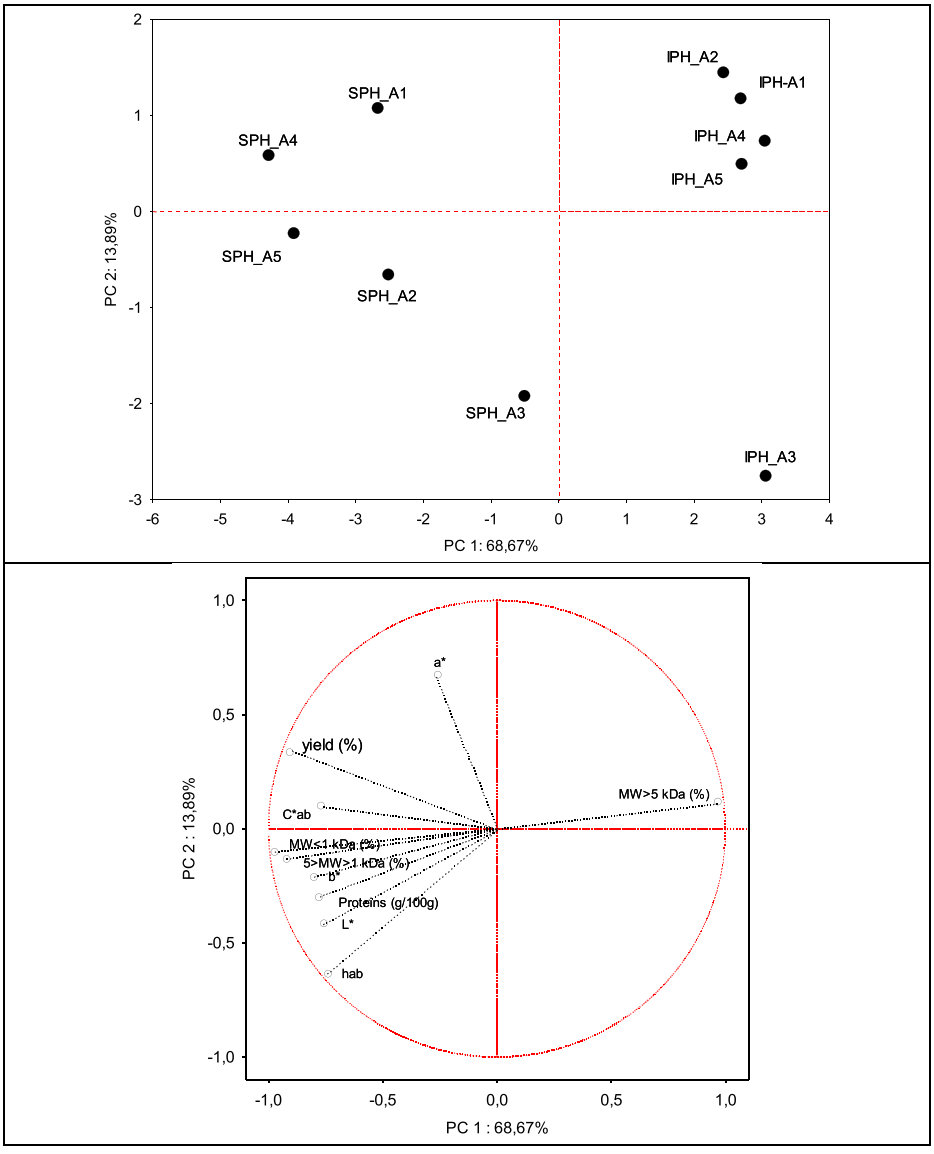

Fig. 2. Scatterplot of samples (SPH and IPH; A1–A5) and variables (hydrolysates yield, protein content, molecular weight distribution and CIELAB parameters) plot onto the plane defined by the first two principal components.

variance. Fig. 2 illustrates the samples to the plane defined by the two first principal components (PCs), which explained 82.6% of the total variability. PC-1 divided the samples into two groups: SPHs and IPHs. IPHs were located on the positive axis of PC-1 and presented lower scores than SPHs for all parameters except for the percentage of the fraction with MW larger than >5 kDa. PC-2 separated the products obtained using assay A3 with negative values, i.e., the hydrolysis of APC, mainly due to the chromatic characteristics (L^*, b^*, and h_{ab}), the percentage of proteins and the low-MW fraction.

Overall, SPH obtained using assays A1 and A2 presented the highest protein purity, MW distribution, and lightness and similar hydrolysates yield. Although IPH from assay A3 showed the best results compared to the rest of IPH samples, its protein purity was not significantly higher than that of IPH from assay A1 and A2 that presented a 15-fold higher hydrolysates yield. In addition, assay A3 was more time-consuming than assay A1, which is less advantageous economically. Similarly, since higher quantity of enzyme did not modify any of the studied parameters, and to counteract the eventual decrease of its activity during the process, the use of an amount of enzyme higher than 0.3% would be appropriate. Since only 0.3% and 0.6% of enzyme have been assessed, the assay A2 was selected as the optimum in this study, thus overcoming the disadvantage of using different industrial methods for the SPHs and IPHs.

3.5. Amino acids composition and peptide sequence

Table 3 shows the amino acid composition of SPH and IPH of assay A2, including individual and total amino acids (TAA) contents grouped into sulphurous (Met and Cys), hydrophobic (Ala, Val, Leu, Ile, Pro, Met and Phe) and aromatic (His, Phe, Pro, and Tyr) amino acids. Generally, the main amino acids found in the hydrolysed products corresponded to negative and positive charged residues (Glu and Arg, respectively), polar (Gly) and hydrophobic amino acids, with a less contribution of the aromatic ones. Individually, Glu and Gly were predominant amino acids, which accounted for over 30–35 and 20–25%, respectively. The hydrolysed products were also rich in Arg, Ser, Ala, Val, Asp and Leu (5–7%), whereas the minor amino acids were Thr, His, Lys, Tyr, Phe, Pro and, especially, the sulphur Met and Cys. It is stands out the lack of Trp, Gln and Asn, probably due to the acid hydrolysis conditions used for the amino acids determination that could provoke the destruction and transformation into the corresponding acids (Glu and Asp, respectively) (Tsugita & Scheffler, 1982). The amino acids profile was in agreement with other results found in grape seeds (Igartuburu, del Río, Massanet, Montiel, Pando, & Luis, 1991), and oil seeds (Ugolini et al., 2015).

Table 3
Amino acids content (mg/g protein product) of SPHs and IPHs of assay A2.

	SPH	IPH
Asp	4.19 ± 0.67	8.66 ± 1.38
Thr	2.50 ± 0.80	2.65 ± 0.50
Ser	11.34 ± 1.50	6.98 ± 0.79
Glu	59.52 ± 6.02	31.35 ± 6.31
Gly	31.16 ± 3.82	24.07 ± 5.00
Ala	11.06 ± 0.63	7.83 ± 0.69
Cys	0.87 ± 0.06	0.76 ± 0.23
Val	7.53 ± 0.81	5.46 ± 0.68
Met	1.24 ± 0.31	1.02 ± 0.22
Ile	6.71 ± 0.63	4.06 ± 0.74
Leu	9.99 ± 1.02	6.37 ± 1.14
Tyr	2.95 ± 0.32	1.88 ± 0.38
Phe	4.60 ± 0.73	2.41 ± 0.51
Hist	2.85 ± 0.31	2.86 ± 0.64
Lys	3.95 ± 0.44	2.87 ± 0.65
Arg	9.79 ± 0.94	7.84 ± 2.15
Pro	nd	3.36 ± 1.27
TAA	170.3 ± 3.7	109.0 ± 15.6
SAA	2.11 ± 0.28	1.78 ± 0.45
AAA	10.40 ± 1.35	10.52 ± 2.79
HAA	44.08 ± 2.70	32.40 ± 5.24

SPH, soluble protein hydrolysate. IPH, insoluble protein hydrolysate. SAA, Sulphur containing amino acids. AAA, aromatic amino acids. HAA, hydrophobic amino acids. TAA, Total amino acids.

Amino acid sequence of peptides, mass, length, and m/z of SPH and IPH of assay A2 analysed by RP-LC-MS/MS are shown in Table 4. For both fractions, the number of amino acids in the peptide sequence ranged from 7 to 19, with MWs between 787 and 1846 Da. Similar results were reported in hydrolysed rice bran protein (Wattanasiritham, Theerakulkait, Wickramasekara, Maier, & Stevens, 2016) or perilla seed meal protein (Kim, Liceaga, & Yoon, 2019), but no information regarding peptide sequences of hydrolysed grape seed was found. Peptides with a length ranged between 2 and 20 amino acid residues has been described as bioactive peptides, which could cross the intestinal barrier and exert biological effects (Shahidi & Zhong, 2008), and displayed antioxidant activity (Ghribi et al., 2015b). A large number of peptides were detected, coming from 6 to 7 proteins ascribed to *Vitis vinifera*, many of which were common to both products. Similarly, as previously commented, the major amino acids in the peptide sequences corresponded to negative and positive charged residues, polar and hydrophobic amino acids, in both SPH and IPH. The predominant amino acids were Gly, Gln and its respective acid Glu, which were estimated to be 10–15% each one (Table 4). They also stood out a considerable percentage of Asp, and Asn in both SPH and IPH, apart from Ser and Arg in IPH. According Suetsuna, Ukeda, & Ochi (2000), Asp plays an important role in the antioxidant activity by carboxyl groups in their side chains, and Ghribi et al. (2015b) established the peptides containing Asp and Gly with a remarkable antioxidant activity. Moreover, the terminal amino acids were mainly Gln, Arg, Phe and Glu, sitting Gln and Glu at the *N*-terminal in the sequence, and Arg, Gln and Phe at the C-terminal of peptide sequence (Table 4). Arg at the C-terminal has been related to a high antioxidant activity (Guo, Kouzuma, & Yonekura, 2009). These results were in agreement with Ghribi et al. (2015a) who affirmed that Alcalase hydrolyses proteins with broad specificity for peptides bonds, with preference for a large uncharged residues as Gln and Asn.

4. Conclusions

This study is the first attempt at optimising the methodology for obtaining wine protein hydrolysates-based products, both soluble and insoluble, from a residue of the grape pomace industry. Based on the protein content, hydrolysates yield, colour, and MW distribution, the obtained products by hydrolysis of a protein concentrate using a content of Alcalase up to 0.3% have been selected as optimum. The use of protein fractions with different MW profiles and amino acid peptides sequences supposes a non-animal alternative to traditional fining and stabilizers agents, allergenic and non-grape derived, for a wine sensory improvement. To bring this in fruition, a further comprehensive study should be made to evaluate the effect of adding the obtained hydrolysed products to wine. Thus, these products could be used as industrial colour stabilisers and fining agents in winemaking, which could be a significant step forward in the field of industrial winemaking concerning colour stabilisation of wines elaborated in warm climates, typically affected by colour loss.

CRediT authorship contribution statement

María Jesús Cejudo-Bastante: Investigation, Writing - original draft. **Melanie Oliva-Sobrado:** Investigation. **M. Lourdes González-Miret:** Writing - review & editing. **Francisco J. Heredia:** Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

Table 4
Amino acid peptide sequence, mass, length and *m/z* of SPH and IPH of assay A2 determined by RP-LC-MS/MS.

Accession number	Peptide sequence	Mass	Length	*m/z*
SPH				
F6HZK3	DTSNDANQLDFQPR	1620.7015	14	811.3631
	EVEEGDVF	904.3814	8	905.3914
	EVFDHNNEQF	1259.5206	10	1260.5326
	EVFDHNNEQFQ	1406.5739	11	1407.5886
	KIREVEEGDVF	1319.6721	11	660.8446
	LLPSYVNAPQLM	1361.6901	12	681.8550
	NIGDPWRADVY	1304.6149	11	653.3170
	NDANQLDFQPR	1317.5948	11	659.8058
	NVFNEEVQQG	1164.4935	10	1165.5015
	QEGGGSEGRGQ	1117.4749	11	559.7408
	QQEGGGSEGRGQ	1171.4854	12	586.7523
	QQQEGGGSEGRGQ	1299.5439	13	650.7800
	TSNDANQLDFQPR	1505.6746	13	753.8441
	VLDTSNDANQL	1189.5463	11	1190.5564
	VLDTSNDANQLDFQPR	1832.8540	16	917.4354
	YVNAPQLM	951.4371	8	952.4476
F6HZK2	DVSNEANQLDFQPR	1633.7219	14	817.8704
	EVFDHNNEQF	1278.5153	10	1279.5229
	EVFDHNNEQFQ	1406.5739	11	1407.5886
	ESSGDNIFSGF	1159.4669	11	1160.4749
	QEGGGSEGRGQ	1117.4749	11	559.7408
	QQEGGGSEGRGQ	1171.4854	12	586.7523
	QQQEGGGSEGRGQ	1299.5439	13	650.7800
	NIGDPWRADVY	1304.6149	11	653.3170
	NEANQLDFQPR	1333.5786	11	667.7980
	STIGAPGSSR	931.4723	10	466.7443
	TIEPNGLLLPSYVNAPQ	1807.9355	17	904.9769
	TIGAPGSSRSE	1060.5149	11	531.2654
	VLDVSNEANQLDFQPR	1845.8744	16	923.9482
A5C7L5	ERVVPVNPA	961.5345	9	962.5438
	RLHPGIDVSHP	1226.6520	11	409.8921
	RLHPGIDVSHPL	1339.7361	12	447.5865
D7U302	DISNDANQLDFQPR	1634.7059	14	818.3615
	NDANQLDFQPR	1318.5790	11	660.2969
F6HI56	PVALPDEFQPF	1258.6233	11	1259.6354
IPH				
F6HZK3	ANQLDFQPR	1088.5250	9	545.2712
	DANQLDFQPR	1203.5520	10	602.7855
	DAQQLAEAF	991.4611	9	496.7402
	DAQQLAEAFNVDVQ	1546.7263	14	774.3726
	DTSNDANQLDFQPR	1620.7015	14	811.3604
	EGRGQESSGDNIF	1395.5902	13	698.8035
	ESSGDNIF	849.3505	8	850.3580
	ESSGDNIFSGF	1140.4723	11	1141.4828
	ESSGDNIFSGFDAQ	1472.6055	14	737.3116
	ESTIAPPGSS	944.4451	10	945.4554
	ESTIAPPGSSRSE	1316.6207	13	659.3211
	EVFDHNNEQF	1259.5206	10	1260.5323
	EVFDHNNEQFQ	1406.5739	11	704.2975
	FIYNNGDRQL	1240.5724	10	621.2952
	FIYNNGDRQLIVV	1552.7773	13	777.3959
	GFEYVAIK	925.4908	8	463.7541
	GGLQAVLPPRGQ	1191.6724	12	596.8462
	GRGQESSGDNIF	1266.5476	12	634.2822
	IGDPWRAD	928.4402	8	465.2290
	IGDPWRADVY	1222.5618	10	612.2875
	IYNNGDRQLIV	1306.6405	11	654.3295
	IYNNGDRQLIVV	1405.7089	12	703.8633
	LLPSYVNAPQ	1100.5865	10	551.3020
	MITGCPETF	1055.4304	9	528.7305
	NDANQLDFQPR	1316.6108	11	659.3145
	NIGDPWRADVY	1304.6149	11	653.3162
	NPQNEFQ	876.3613	7	877.3704
	QEGGGSEGRGQ	1043.4268	11	522.7223
	QESSGDNIFSGF	1269.5149	12	635.7656
	QLDFQPR	902.4610	7	452.2372
	QNIGDPWRA	1038.4883	9	520.2532
	QQEGGGSEGR	986.4053	10	494.2142
	QQEGGGSEGRGQ	1171.4854	12	586.7517
	QQEGGGSEGRGQE	1300.5280	13	651.2729
	QQQEGGGSEGR	1114.4639	11	558.2403

Table 4 (*continued*)

Accession number	Peptide sequence	Mass	Length	*m/z*
	QQQEGGGSEGRGQ	1299.5439	13	650.7805
	RGQESSGDNIF	1208.5421	11	605.2810
	SGFDAQQLAEAF	1282.5829	12	642.3001
	STIAPPGSSRSE	1187.5782	12	594.7981
	TSNDANQLDFQPR	1505.6746	13	753.8469
	VFDHNNEQF	1149.4727	9	575.7465
	VLDTSNDANQL	1189.5463	11	595.7833
	VLDTSNDANQLDFQPR	1832.8540	16	917.4351
	VNAPQLM	787.3898	7	788.3988
	VVQQQGQNVFNEE	1517.7111	13	759.8664
	YEETICSL	988.4059	8	495.2034
	YVNAPQLM	950.4531	8	476.2359
F6HZK2	ANQLDFQPR	1088.5250	9	545.2712
	DAQQLAEAF	991.4611	9	496.7402
	DAQQLAEAFNVDVQ	1546.7263	14	774.3726
	DVSNEANQLDFQPR	1633.7219	14	817.8701
	EANQLDFQPR	1217.5676	10	609.7928
	EGRGQESSGDNIF	1394.6062	13	698.3130
	ESSGDNIF	849.3505	8	850.3580
	ESSGDNIFSGF	1140.4723	11	1141.4828
	ESSGDNIFSGFDAQ	1472.6055	14	737.3116
	ESTIGAPGSS	886.4032	10	887.4009
	EVEEGDAF	916.3426	8	917.3550
	EVFDHNNEQF	1259.5206	10	1260.5323
	EVFDHNNEQFQ	1407.5579	11	704.7881
	GFEYVAIK	925.4908	8	463.7541
	GRGQESSGDNIF	1266.5476	12	634.2822
	GYDLPVLQ	903.4702	8	904.4797
	IGDPWRAD	928.4402	8	465.2290
	IGDPWRADVY	1222.5618	10	612.2875
	IREVEEGDAF	1163.5458	10	582.7828
	ISGCPETF	884.3586	8	443.1779
	LLPSYVNAPQ	1100.5865	10	551.3020
	NEANQLDFQPR	1333.5786	11	667.7996
	NIGDPWRADVY	1304.6149	11	653.3162
	NPQNEFQ	876.3613	7	877.3704
	QEGGGSEGRGQ	1043.4268	11	522.7223
	QESSGDNIFSGF	1269.5149	12	635.7656
	QFLGDQHQ	972.4301	8	487.2240
	QKPSNRIQ	952.5090	8	477.2626
	QLDFQPR	902.4610	7	452.2372
	QNIGDPWRA	1038.4883	9	520.2532
	QQEGGGSEGR	986.4053	10	494.2142
	QQEGGGSEGRGQ	1171.4854	12	586.7517
	QQEGGGSEGRGQE	1300.5280	13	651.2729
	QQQEGGGSEGR	1114.4639	11	558.2403
	QQQEGGGSEGRGQ	1299.5439	13	650.7805
	RGQESSGDNIF	1208.5421	11	605.2810
	SGFDAQQLAEAF	1282.5829	12	642.3001
	STIGAPGSSRSE	1147.5469	12	574.7826
	TIEPNGLLLPS	1153.6230	11	577.8195
	TIEPNGLLLPSYVN	1529.7977	14	765.9076
	TLAGNLSLM	934.4794	9	468.2483
	VFDHNNEQF	1149.4727	9	575.7465
	VLDVSNEANQL	1200.5986	11	601.3071
	VLDVSNEANQLDFQPR	1846.8584	16	924.4371
	VVQQQGQTVANEE	1428.6844	13	715.3532
	YEETICSL	988.4059	8	495.2034
	YTIEPNGLLLPS	1316.6864	12	659.3508
A5C7L5	ANSMVQPRPG	1072.4971	10	537.2566
	ERVVPVNPA	961.5345	9	481.7755
	FVDGGSNPKAPIIL	1426.7820	14	714.4014
	INERVVPVNPAL	1320.7401	12	661.3795
	LFACAPSSLAQK	1276.6486	12	639.3209
	NSMVQPRPG	1001.4600	9	501.7380
	RLHPGIDVSHPL	1339.7361	12	447.5878
	RLHPGIDVSHP	1226.6520	11	409.8930
D7U302	ANQLDFQPR	1089.5090	9	545.7641
	DANQLDFQPR	1203.5520	10	602.7855
	DISNDANQLDFQPR	1633.7219	14	817.8701
	EVQEGDVF	922.3920	8	923.4019
	ISGCPETF	884.3586	8	443.1779
	NDANQLDFQPR	1317.5948	11	659.8066
	QLDFQPR	885.4344	7	443.7249
F6HI56	ALPDEFQPF	1062.5022	9	532.2589

(*continued on next page*)

Table 4 (*continued*)

Accession number	Peptide sequence	Mass	Length	*m/z*
	SGMFPFPF	944.4102	8	473.2143
	SQREESGMF	1085.4447	9	543.7311
	SQREESGMFPF	1329.5659	11	665.7927
	SQREESGMFPFPF	1573.6871	13	787.8524
	PVALPDEFQPF	1258.6233	11	630.3207
F6GTY5	KKQPVEPTEPY	1315.6659	11	658.8428

SPH, soluble protein hydrolysate. IPH, insoluble protein hydrolysate.

the work reported in this paper.

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Appendix A. Supplementary data

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