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Exploration of the antioxidant and antimicrobial capacity of two sunflower protein concentrate films with naturally present phenolic compounds

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ABSTRACT

The aim of this study was to assess the potential activation of sunflower protein films with antioxidant and antimicrobial properties conferred by the phenolic compounds of sunflower seeds, which remain associated to the proteins used as starting material for film preparation. Two sunflower protein concentrates obtained from the residual pellet of oil industry were used, which had different content of phenolic compounds, mainly chlorogenic and caffeic acids. The film-forming dispersions and the films obtained were analyzed regarding their antioxidant properties (using ABTS, FRAP and PCL assays) and their antimicrobial properties (by agar disk diffusion tests). Phenolic compounds conferred important antioxidant properties to both dispersions and films, this activity being dependent on their content and their free or protein-bound nature. These compounds, however, did not confer their characteristic antimicrobial properties reported in previous studies, possibly due to their interaction with proteins and the pH of the film-dispersions. Since the antioxidant activity of phenolic compounds was preserved during the protein concentration process (inherent to the film formation), these protein matrices may be considered useful for protecting these bioactive compounds.

1. Introduction

Several proteins of agricultural origin have been studied regarding their ability to form edible and/or biodegradable films (Cuq, Gontard, & Guilbert, 1998; Gennadios, 2002), including sunflower proteins (Ayhllon-Meixueiro, Vaca-Garcia, & Silvestre, 2000; Orliac, Rouilly, Silvestre, & Rigal, 2003; Rouilly, Mériaux, Geneau, Silvestre, & Rigal, 2006; Salgado, Molina Ortiz, Petrucelli & Mauri, 2010). These macromolecules can be extracted from the residual pellet of the oil industry (sunflower oilcake) using environment-friendly simple methods, thus constituting economic starting materials. Phenolic compounds naturally present in sunflower seeds, mainly chlorogenic and caffeic acids, are also extracted during these procedures. While it is possible to reduce the content of these compounds in protein products by modifying the extraction procedure (González-Pérez et al., 2002; Salgado, Molina Ortiz, Petruccelli, & Mauri, 2011), it is impossible to eliminate them completely due to their interaction with proteins (Prasad, 1990; Sastry & Subramanian, 1984; Salgado et al., 2012; Salgado, Molina Ortiz et al., 2011; Sripad & Narasinga Rao, 1987).

Several studies performed in the last decade have demonstrated that aqueous and alcoholic solutions of these phenolic compounds in pure form have antioxidant and antimicrobial activities, both *in vitro* (Brand-Williams, Cuvelier, & Berset, 1995; Raskin et al., 2002; Rice-Evans, Miller, & Paganga, 1995; Klančnik, Guzej, Hadolin Kolar, Abramovič, & Smole Možina, 2009) and *in vivo* (Balasundram, Sundram, & Samman, 2006; Bowles & Miller, 1994; Medina, Gallardo, González, Lois, & Hedges, 2007; Rodriguez de Sotillo, Hadley & Wolf-Hall, 1998). However, these compounds can interact with other components of the alimentary and/or polymeric matrix (mainly proteins and polysaccharides) (Le Bourvellec & Renard, 2012), therefore it is important to determine whether they maintain their bioactivity in a given product or material. This issue has begun to be studied in recent years (Rawel & Rohn, 2010; Sivarooban, Hettiarachchy, & Johnson, 2008; Salgado, Molina Ortiz, Petruccelli, & Mauri, 2010; Salgado, Fernández, Drago, & Mauri, 2011; von Staszweski, Pilosof, & Jagus, 2011).

In a previous study, Salgado et al. (2010) reported the formation of biodegradable films from sunflower protein isolates having different concentrations of phenolic compounds and observed that

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the concentration of these compounds, which varied slightly among protein isolates (from 1.82% to 2.51%) did not affect the physicochemical properties of the films-thickness, density, water content, water vapor permeability, mechanical properties, glass transition temperature, and type of interactions involved in the protein network formation (which in all cases were mainly hydrogen bonds, hydrophobic interactions, and disulfide bonds)-, but was enough to endow the films with antioxidant properties (measured by the ABTS method) and UV light barrier properties (Salgado et al., 2010).

The aim of the present study was to gain further insight in the antioxidant properties, and to asses the antimicrobial activity, of films prepared from two sunflower protein concentrates with a phenolic content similar or twice of that mentioned above, discussing the potential contribution of interactions between phenolic compounds and proteins.

2. Materials and methods

2.1. Preparation of sunflower protein concentrates

Two sunflower protein concentrates (**SFPC** and **SFPC + IP**) obtained from the sunflower oilcake (**SFOC**) provided by Aceitera Santa Clara (Molinos Río de La Plata, Rosario, Argentina) were employed in this study. Aqueous dispersions (45 L) of the sunflower oilcake (67 g/L) were stirred for 1 h and their pH was adjusted to 9 with 3 mol/L NaOH, using a pH meter (IQ 150, Cientific Instruments, USA). Solid-liquid separation was performed in a basket type centrifuge with filtering material (0.8–1.0 mm pore size) (made at the Institute of Food Technology – ITA-UNL –, Argentine) at 2100× *g* and 20 °C; and the filtrate was collected. The residue was subjected to a second extraction of proteins as described above. The filtrates of both extractions were pooled, the pH was adjusted to 9 with 3 mol/L NaOH, and the mixture was:

i) spray-dried using a Niro Atomiser spray drier with an inlet temperature of 170–190 °C and an outlet temperature of 80–90 °C (Niro Atomiser Production Minor, Denmark) to obtain the sunflower protein concentrate (**SFPC**); or
ii) subjected to isoelectric precipitation by adjusting the pH to 4.5 with 3 mol/L HCl, using a pH meter (IQ 150, Cientific Instruments, USA). The mixture was stirred for 30 min and separation of the precipitate was carried out in a Westfalia centrifuge (Westfalia SAADH 205 model, Germany) at 20 °C. The resulting precipitate was washed and was centrifuged once more under the same conditions described above. This washed precipitate was resuspended in water (approximately 0.5 L/kg precipitate). The suspension was passed through a Manton-Gaulin two-stages homogenizer (Gaulin Corp., USA) with 2×10^5 and 5×10^5 Pa in the first and second stage respectively, the pH was adjusted to 9 with 3 mol/L NaOH, and the solution was spray-dried using a Niro Atomiser spray drier with an inlet temperature of 170–190 °C and an outlet temperature of 80–90 °C (Niro Atomiser Production Minor, Denmark). This product was named as sunflower protein concentrate obtained with isoelectric precipitation (**SFPC + IP**).

2.2. Characterization of sunflower protein concentrates

Chemical composition: Moisture and ash values were determined by gravimetric measure (AOAC 935.29 and AOAC 923.03, 1995). Protein content was determined by the Kjeldahl method (AOAC 920.53, 1995) using 5.55 as nitrogen-to-protein conversion factor. Phenolic compounds were measured by UV spectrophotometry at 324 nm as described by Moores, McDermott, and Wood (1948), using chlorogenic acid (Chemika Fluka, Germany) as the standard. Extraction of lipids was performed in a Soxhlet apparatus using *n*-hexane (Cicarelli, Argentine) as solvent and its quantification was carried out by gravimetric measure (AOAC 922.06, 1995). Total soluble sugars were quantified by the spectrophotometric anthrone method at 620 nm in 64% v/v sulfuric acid, using glucose (Sigma Aldrich Chemical Co., St. Louis, USA) as the standard (Loewus, 1952). The percent content of fibers was calculated by difference. All determinations were performed at least in duplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The polypeptide composition of sunflower protein concentrates was analyzed by SDS-PAGE using a 0.12 g polyacrylamide/mL separating gel and a 0.04 g polyacrylamide/mL stacking gel in a minilabs system (Bio-Rad Mini-Protean II Model) (Laemmli, 1970). Protein molecular weights were estimated using low MW markers (94, 67, 43, 30, 20.1 and 14.4 kDa) (Pharmacia, Amersham, England). Gel images were analyzed with the ImageJ software (Bethesda, MD: US National Institute of Health).

Differential Scanning Calorimetry (DSC): A TA Instrument DSC Q100 V9.8 Build 296 (New Castle, Del., USA) was used for these studies. Hermetically sealed aluminum pans containing 10–15 mg of sunflower protein concentrates dispersed in distilled water (0.2 g/mL) were prepared and scanned at 10 °C/min over the 20–120 °C range. Denaturation enthalpies (ΔH) and peak temperatures (Td, in °C) were taken from the corresponding thermograms (Universal Analysis V4.2E, TA Instruments, New Castle, USA). Enthalpy values (ΔH) were expressed as J/g protein, taking into account the dry weight (determined by perforating the pans and heating overnight at 105 °C) and the protein content of sample (Molina, Petruccelli, & Añón, 2004). All assays were performed in duplicate.

Surface Hydrofobicity (Ho) was determined according to the method described by Kato and Nakai (1980) in a digital fluorometer Perkin–Elmer model 2000 (Norwalk, CT, USA), using 8-aniline-1-naphthalene sulfonate (ANS, Aldrich Chemical Company, Inc., USA) as probe ($\lambda_{excitation}$ = 364 nm and $\lambda_{emission}$ = 475 nm). All determinations were performed in quadruplicate.

2.3. Preparation of films

Film-forming dispersions were prepared dispersing **SFPC** or **SFPC + IP** (5% w/v) and glycerol (1.5% w/v, Anedra, Argentine) in distilled water. Dispersions were agitated in a magnetic stirrer for 30 min at room temperature, their pH was adjusted to pH 11 with 2 mol/L NaOH, and they were stirred again for 30 min. Films were prepared by casting, for which 12.5 mL of this filmogenic dispersion were poured on polystyrene Petri dishes (64 cm^2) and then dehydrated at 60 °C for 5 h in an oven with air flow circulation (Yamato, DKN600, USA). Dry films were conditioned for 48 h at 20 °C and 58% relative humidity (RH) in desiccators with saturated solutions of NaBr before being peeled from the casting surface for characterization.

2.4. Characterization of films

Film thickness was measured by a digital coating thickness gauge (Check Line DCN-900, USA). The thickness was measured in 9 randomly selected points on each film and then an average value was calculated.

Moisture Content (MC) of the films was determined after drying them in an oven at 105 °C for 24 h. Small specimens of films collected after conditioning were cut and placed on Petri dishes that were weighed before and after oven drying. MC values were determined in triplicate for each film, and calculated as the percentage of weight loss relative to the original weight.

Water Solubility (WS) of the films was determined following the method described by Gontard, Duchez, Cuq, and Guilbert (1994) with slight modifications. Film portions were weighed (diameter: 2 cm; $P_o \approx 0.10-0.15$ g) and placed in Erlenmeyer flasks (250 mL) with 50 mL of distilled water (0.02% w/v sodium azide). Flasks were sealed and shaken at 100 rpm (Ferca, TT400 model, Argentine) for 24 h at 20 °C. The solution was then filtered through Whatman n° 1 filter paper (previously dried and weighed) to recover the remaining undissolved film, which was desiccated at 105 °C for 24 h (P_f). WS values were determined in triplicate for each film, and calculated using the following equation:

$$WS = \frac{P_o \cdot (100 - MC) - P_f}{P_o \cdot (100 - MC)} \cdot 100$$

2.5. Release of protein and phenolic compounds in water and protein-phenolic compounds interactions

The content of *sunflower proteins released* from the film matrix in *WS* test was quantified by the Bradford method (Bradford, 1976) using bovine serum albumin (Sigma Aldrich Chemical Co., St. Louis, USA) as the standard.

Total phenolic compounds content in both filmogenic dispersions and *WS* test supernatants was determined by UV spectrophotometry at 324 nm (Moores et al., 1948) using chlorogenic acid (Chemika Fluka, Germany) as the standard.

To determine the amount of *phenolic compounds complexed to sunflower proteins*, aliquots of filmogenic dispersions and *WS* test solutions were mixed with 20% w/v trichloroacetic acid (TCA, Anedra, Argentine) and centrifuged for 10 min at 12000× *g* and 20 °C to separate the complexes formed. The content of phenolic compounds in neutralized supernatants (free soluble phenolic compounds), and in resuspended-neutralized precipitates (soluble phenolic compounds–proteins complexes) was determined as described above. Results were expressed as percent of water-soluble proteins and phenolic compounds with respect to the total content of these compounds present in the corresponding films. Determinations were performed in quadruplicate.

2.6. Antioxidant properties

The antioxidant capacity of the filmogenic dispersions and the supernatants obtained in *WS* test of the resulting protein films were characterized by its cationic radical scavenging ability (ABTS assay), its ferric ion reducing capacity (FRAP assay), and its capacity to quench superoxide anion radicals (PCL assay). The methods used for the ABTS and FRAP assays were previously described by Gómez-Estaca, Giménez, Gómez-Guillén, and Montero (2009). Results were expressed as mg of Vitamin C Equivalent Antioxidant Capacity (VCEAC) per mL of filmogenic dispersion, or per g of film (ABTS assay), and in mmol $FeSO_4.7H_2O$ equivalents per mL of filmogenic dispersion, or per g of film (FRAP assay), based on standard curves for each compound.

PCL assays: The luminol-photochemiluminescence assay was carried out in a PHOTOCHEM® (Analytik Jena AG, Germany) system with the kits of antioxidant capacity of water-soluble substances (ACW) and antioxidant capacity of lipid-soluble substances (ACL), where the luminol plays a double role as the photosensitizer and the radical detecting agent. The hydrophilic and lipophilic antioxidants were measured using the ACW kit (1.5 mL of buffer solution pH 10.5, 1 mL of water, 25 μL of photosensitizer and 10 μL of the sample solution) and the ACL kit (2.3 mL of methanol, 200 μL of buffer solution, 25 μL of photosensitizer and 10 μL of the sample solution) respectively, following the instructions of the manufacturer. Calibration curves were performed with ascorbic acid and Trolox® as standards for ACW and ACL respectively. Results were expressed as μmol of standard per mL of filmogenic dispersion, or per mg of film. Three replicates were made for each test sample.

2.7. Antimicrobial properties

The antimicrobial activity of the filmogenic dispersions and the resulting protein films were determined by the agar disk diffusion method against 26 microbial strains, as previously described (Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010). The strains, selected because of its importance in human health (either probiotics or pathogens) or for being responsible for food spoilage, were obtained from the Spanish Type Culture Collection (CECT): *Lactobacillus acidophilus* CETC 903, *Salmonella cholerasuis* CECT 4300, *Listeria innocua* CECT 910, *Citrobacter freundii* CECT 401, *Escherichia coli* CECT 515, *Shigella sonnei* CECT 4887, *Pseudomonas aeruginosa* CECT 110, *Yersinia enterocolitica* CECT 4315, *Brochothrix thermosphacta* CECT 847, *Staphylococcus aureus* CECT 240, *Bacillus cereus* CECT 148, *Listeria monocytogenes* CECT 4032, *Clostridium perfringens* CECT 486, *Aeromonas hydrophila* CECT 839T, *Photobacterium phosphoreum* CECT 4192, *Shewanella putrefaciens* CECT 5346T, *Pseudomonas fluorescens* CECT 4898, *Vibrio parahaemolyticus* CECT 511T, *Bacillus coagulans* CECT 56, *Bifidobacterium animalis* subespecie *lactis* DSMZ 10140, *Bifidobacterium bifidum* DSMZ 20215, *Enterococcus faecium* DSM 20477, *Lactobacillus helveticus* DSM 20075, *Debaryomyces hansenii* CECT 11364, *Aspergilus niger* CECT 2088, *Penicilium expansum* DSMZ 62841. After incubation the inhibition area around the disks-considered the marker of antimicrobial activity-was measured with Adobe Acrobat® Reader 6 Professional software. Results were expressed as percentage of growth inhibition respect to the total plate surface. Each determination was performed in duplicate.

2.8. Statistical analysis

Results were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA). Means were tested with the Tukey's HSD (Honestly Significant Difference) test for paired comparison, with a significance level $\alpha = 0.05$, using the SPSS® software (SPSS Statistical Software version 15.0, USA).

3. Results and discussion

3.1. Characterization of raw materials

Chemical composition of sunflower oilcake (**SFOC**) and sunflower protein concentrates (**SFPC** and **SFPC + IP**) are shown in Table 1. **SFOC** had high content of proteins (31.7% d.b.), fibers and carbohydrates, and presented a minimal content of lipids (<1.0% d.b.). A particular characteristic of the **SFOC**, as compared to other sources of vegetable proteins (e.g. soy) was its high content of phenolic compounds (2.7% d.b.), mainly chlorogenic and caffeic acids. Žilić et al. (2010) also reported the presence in smaller quantities (<0.02% d.b.) of ferulic acid, rosmarinic acid, myricetin and rutin in sunflower seeds. The detailed percent chemical composition is similar to that reported in studies on similar products (Ayhllon-Meixueiro et al., 2000; Rouilly, Orliac, Silvestre, & Rigal, 2006; Vioque, Sánchez-Vioque, Pedroche, Yust, & Millán, 2001).

Sunflower protein concentrates (**SFPC** and **SFPC + IP**) employed in this study differed in their chemical composition as shown in Table 1. These variations were due to the different preparation procedures employed. **SPFC** preparation is based on the solubilization of proteins (and other soluble components) in alkaline aqueous medium, followed by dehydration by spray drying. For the preparation of

Table 1
Chemical composition of sunflower oilcake (**SFOC**) and sunflower protein concentrates (**SFPC** and **SFPC + IP**). Recoveries of proteins and phenolic compounds for each procedure.

Sample	Chemical composition (%)							Recovery of (%)***	
	Proteins*	Phenolic compounds*	Ashes*	Lipids*	Carbohydrates*	Fibers*, **	Moisture	Proteins	Phenolic compounds
SFOC	31.7 ± 0.1^{a}	2.7 ± 0.1^{b}	8.0 ± 0.4^{b}	0.9 ± 0.1^{a}	23.2 ± 1.3^{a}	33.5	11.0 ± 0.9^{c}	–	–
SFPC	41.4 ± 2.9^{b}	5.4 ± 0.3^{c}	11.4 ± 0.1^{c}	1.1 ± 0.1^{a}	19.3 ± 1.1^{b}	21.4	6.3 ± 0.1^{b}	41.3 ± 0.4^{b}	64.3 ± 0.5^{b}
SFPC + IP	70.4 ± 0.8^{c}	2.5 ± 0.1^{a}	4.0 ± 0.1^{a}	1.0 ± 0.1^{a}	4.9 ± 0.6^{c}	17.2	4.9 ± 0.4^{a}	16.6 ± 0.2^{a}	7.1 ± 0.6^{a}

Reported values for each protein product are means ± standard deviation. *Percentages expressed in dry basis. **The content of fibers was calculated by difference. ***The content of proteins or phenolic compounds in the sunflower oilcake was considered 100%. Different letters (a, b, c) in the same column indicate significant differences ($p < 0.05$) according to Tukey's test.

SFPC + IP, in contrast, an isoelectric precipitation (in aqueous medium at pH 4.5) and a resuspension step were added between the protein solubilization and dehydration steps in order to enrich the product in proteins and to reduce the content of other components soluble in acid aqueous medium, such as phenolic compounds, minerals, fibers and carbohydrates.

SPFC was the product with the lowest protein content (41.4%) and the highest content of phenolic compounds (5.4%), minerals (ashes), carbohydrates, fibers and moisture ($p < 0.05$). During its preparation it was possible to retain 41.3% of proteins and 64.3% of the phenolic compounds present in the **SFOC** (Table 1). Although **SPFC + IP** was enriched in proteins at the expense of other components, 16.6% of proteins and 7.1% of phenolic compounds originally present in the **SFOC** were retained during its preparation (Table 1), these protein recoveries are typical for these type of procedures (Salgado et al., 2012). In particular, phenolic compounds retained in this process would be those that interact with proteins even in solution, since they coprecipitate with proteins.

Depending on the extraction procedure, the total polysaccharide content in defatted sunflower meal may vary from 9 to 31% (Düsterhöft, Engels, & Voragen, 1993; González-Pérez et al., 2002; Sabir, Sosulski, & Hamon, 1975; Sodini & Canella, 1977). Many sugars present in sunflower meal, namely glucose, arabinose, uronic acid, galactose and to a lesser extent, xylose, manose, rhamnose and fucose, were also found in the corresponding protein isolates (Düsterhöft et al., 1993; González-Pérez et al., 2002). Sabir et al. (1975) found the principal polysaccharides in water-soluble fraction from sunflower flour to be arabinogalactans. Moreover, they reported an arabinose to galactose ratio of 8:1 in the analysis of the constituent sugars of the resulting protein concentrate extracted with dilute alkali. Natural polysaccharides do not always exist singly in plants, but conjugate with other components, including protein, and therefore, they may act as a whole during protein isolation (Chen, Zhang, Qu, & Xie, 2008). Furthermore, the formation of reactive dicarbonyl sugar (glyoxal) and arabinose as intermediates in the autoxidative glycosylation and crosslinking of proteins by glucose may contribute greatly to protein modification (Wells-Knecht, Zyzak, Litchfield, Thorpe, & Baynes, 1995).

It is worth noting the high proportion of fibers present in **SPFC** (21.4%) and **SPFC + IP** (17.2%). These components are constituted mostly by residual cell wall materials such as nonstarch polysaccharides (cellulose, 4-*O*-methylglucuronoxylans, pectic compounds) and ligno-cellulosic materials (Düsterhöft et al., 1993). Rouilly, Orliac et al. (2006) reported 37.3% of ligno-cellulosic fibers in sunflower oilcake distributed as follows 22.3% cellulose, 5.2% lignin and 9.8% hemicellulose. None of the procedures assayed in the present study resulted in the complete removal of these compounds.

3.2. Characterization of sunflower proteins

The polypeptide composition of **SFPC** and **SFPC + IP** as determined by SDS-PAGE analysis (Fig. 1) did not differ significantly except for a higher intensity of all bands for **SFPC + IP** due to the higher protein concentration of this sample. Under non-reducing conditions (lanes 1 and 2) both sunflower protein concentrates contained mainly 11S globulins (with α-β subunits of molecular mass between 55 and 65 kDa), aggregates of high molecular weight (HMWA, higher than 94 kDa), and lower levels of 2S albumins (Alb, molecular weight between 14 and 18 kDa). Under reducing conditions (lanes 3 and 4) the α–β subunits dissociated into acidic (α) and basic (β) polypeptides (30–40 kDa and 20–30 kDa, respectively) in agreement with a previous report by Molina et al. (2004), and the HMWA disappeared completely from protein samples, suggesting that they were stabilized by disulfide bonds. Both protein samples also showed the presence of bands corresponding to 2S albumins (Alb).

Proteins present in **SFPC** exhibited a higher degree of denaturation (lower ΔH), higher thermal stability (higher Td), and lower surface hydrophobicity (Ho) than those present in **SFPC + IP** ($p < 0.05$) (Table 2). These results may be attributed to the fact that extractable compounds that can interact weakly with proteins are removed during the isoelectric precipitation step. It is known that phenolic compounds can interact with sunflower proteins through weak bonds such as hydrophobic, electrostatic and hydrogen bond interactions, but also through covalent links (González-Pérez & Vereijken, 2008; Rawel & Rohn, 2010). In addition, minerals and

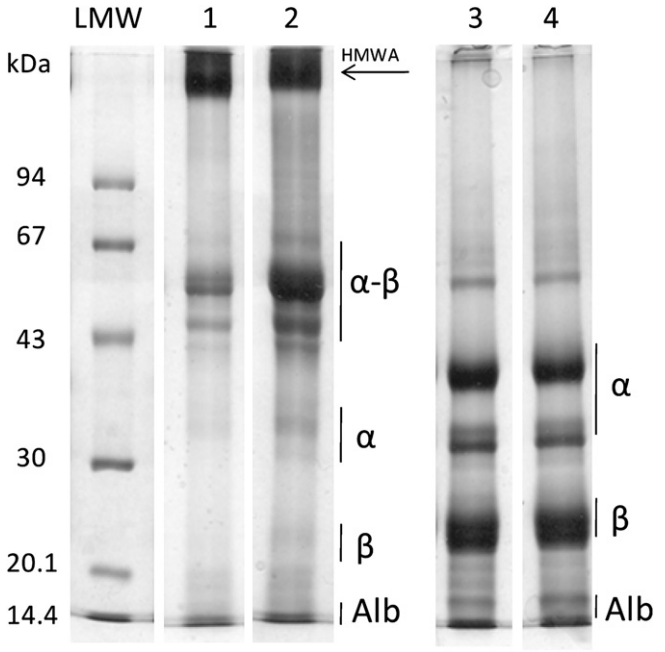

Fig. 1. SDS-PAGE electrophoretic patterns under non-reducing (lanes 1 and 2) or reducing conditions (lanes 3 and 4, β-mercaptoethanol added) of sunflower protein concentrates. Samples: **SFPC** (lanes 1 and 3), **SFPC + IP** (lanes 2 and 4). **LWM**: low molecular weight markers.

Table 2
Denaturation temperatures and enthalpies (Td, ΔH), and surface hydrophobicity (Ho) of sunflower protein concentrates (**SPFC** and **SFPC + IP**).

Sample	Td (°C)	ΔH (J/g protein)	Ho (UA.ml/mg)
SFPC	102.3 ± 0.1^{b}	4.2 ± 0.2^{a}	29.1 ± 0.3^{a}
SFPC + IP	100.1 ± 1.6^{a}	5.4 ± 0.3^{b}	50.4 ± 0.3^{b}

Reported values for each protein product are means ± standard deviation. Different letters (a, b) in the same column indicate significant differences ($p < 0.05$) according to Tukey's test.

carbohydrates interact with proteins through electrostatic and hydrogen bond interactions, and the second may also interact through oxidative or non-oxidative covalent crosslinks inducing protein glycation, glycosylation or glycoxylation (Fennema, 2005; Wells-Knecht et al., 1995). Therefore, removal of these components may lead to conformational changes in proteins and to the establishment of new interactions between their polypeptide chains that would stabilize macromolecules present in **SFPC + IP**, thus increasing their denaturation enthalpy and surface hydrophobicity, with a slight reduction of their thermal stability.

3.3. Water solubility of films

For certain applications in which the film is not used as a physical barrier for food protection the ability to solubilize upon contact with an aqueous or highly humid environment is critical for allowing the release of bioactive compounds. The film water solubility is directly related with the structural properties of proteins and the presence of other non-proteinaceous components in the films, mainly phenolic compounds and carbohydrates, but may be also affected by other factors such as the thickness and the water content of the film. In this sense, the resulting sunflower protein films (**SFPC** and **SFPC + IP**) showed very high water solubility, with no significant differences ($p > 0.05$) in thickness as shown in Table 3. However, the **SFPC** films had higher moisture content (MC) and higher water solubility (WS) than **SFPC + IP** films ($p < 0.05$) (Table 3). These differences could be attributed to the different chemical composition of the raw materials employed (see Table 1), mainly carbohydrates, minerals and phenolic compounds, which could contribute to an increased hygroscopicity of the films developed. **SFPC + IP** exhibited a higher content of proteins (about 1.8 fold higher) and surface hydrophobicity than **SFPC**. On the other hand, the residual phenolic compounds and carbohydrates present in **SFPC + IP** were probably strongly linked to proteins since they were not completely depleted during its preparation. Altogether, these factors lead to the formation of a protein film matrix less soluble in water (lower WS) ($p < 0.05$).

In agreement with the high WS of both films, the percent release of proteins and phenolic compounds to the aqueous phase were also very high (Table 3). While no significant differences were observed between both films regarding the proportion of released phenolic compounds (~98%), the percentage of released proteins was significantly lower ($p < 0.05$) for the **SFPC + IP** film (94.2% vs. 97.1%), suggesting that the lower film solubility is due to a higher degree of protein aggregation. It must be also taken into account that **SFPC** has a high content of components such as carbohydrates and minerals that, although may be occluded in the protein matrix, are highly soluble in water (contributing to increase WS values) ($p < 0.05$) and also have high affinity for water (leading to higher MC values) ($p < 0.05$). Carbohydrates are also present to a lesser extent in **SFPC + IP**, but in this case they are more likely involved in covalent crosslinking with proteins.

Table 3
Thickness, moisture content, water solubility of whole films obtained from sunflower protein concentrates (**SFPC** and **SFPC + IP**), and percent of protein and phenolic compounds released in water with respect to the total content of protein and phenolic compounds present in the corresponding films.

Sample	Film thickness (μm)	Moisture content (%)	Water solubility (%)	Protein release (%)	Phenolics release (%)
SFPC	68 ± 9^{a}	34.1 ± 2.0^{a}	96.9 ± 0.7^{a}	97.1 ± 2.0^{a}	98.1 ± 2.1^{a}
SFPC + IP	70 ± 10^{a}	28.6 ± 1.5^{b}	93.2 ± 1.7^{b}	94.2 ± 2.8^{b}	98.2 ± 3.0^{a}

Reported values for each protein product are means ± standard deviation. Different letters (a, b) in the same column indicate significant differences ($p < 0.05$) according to Tukey's test.

3.4. Antioxidant properties

Table 4 shows the antioxidant capacity of the filmogenic dispersions and the water-soluble fraction from the corresponding sunflower protein films as measured by ABTS, FRAP and PCL assays. Both **SFPC** filmogenic dispersions and film water-soluble fractions exhibited significantly higher values ($p < 0.05$) than their **SFPC + IP** counterparts in all the three assays, indicating a greater antioxidant capacity by either the free radicals scavenging mechanism (ABTS and PCL) or by the ferric ion reducing mechanism (FRAP). These results could be largely attributed to differences in the composition of the raw materials (see Table 1). The high antioxidant capacity of sunflower phenolic compounds, mainly chlorogenic acid, and to a lesser extent caffeic acid, has been well documented (Velioglu, Mazza, Gao, & Oomah, 1998). According to a previous work carried out by Salgado, Molina Ortiz et al. (2011) using the same starting material, the phenolic compounds present in both studied protein concentrates would correspond mainly to chlorogenic acid (around 75%) and caffeic acid (around 19%). In addition, the presence of carbohydrates in **SFPC** and **SFPC + IP**, either in the form of single sugars, polysaccharides or even polysaccharide conjugates, may contribute also to their reducing and radical scavenging capacities (Chen et al., 2008, 2011).

The measured antioxidant properties do not show proportionality with respect to the total phenolic content, which was two-fold greater in **SFPC** than in **SFPC + IP**. This effect was particularly evident during the determination of the quenching of superoxide radical activity (in the PCL assay), especially in relation with the water-soluble substances (PCL–ACW), whose activity was about 25-fold lower in **SFPC + IP** than in **SFPC** (both filmogenic dispersions and films). In addition, the antioxidant activity of the lipid-soluble substances (PCL–ACL) in both samples (**SFPC + IP** and **SFPC**) is consistent with the presence of residual lipids in both protein concentrates (see Table 1). It is important to note that the phenolic compounds present in both preparations, showed antioxidant activity in a wide range of conditions including aqueous solutions at neutral pH (in ABTS), acid pH (FRAP) and alkaline pH (PCL–ACW), and media with hexane (PCL–ACL). However, the antioxidant activity of phenolic compounds could be decreased by interactions with proteins. During protein extraction in alkaline medium, chlorogenic acid and other phenolic compounds are oxidized to *o*-quinones and form covalent linkages with proteins (Sodini & Canella, 1977). Phenolic compounds may also react non-covalently with proteins via hydrogen-bonding, ionic and hydrophobic interactions (Le Bourvellec & Renard, 2012; Saeed & Cheryan, 1989).

To evaluate a potential interaction between phenolic compounds and proteins in sunflower concentrates, both in filmogenic dispersions and water-soluble fractions of films, proteins were precipitated with TCA (10% w/v), and the content of phenolic compounds in each of the resulting fractions (TCA-soluble and TCA-insoluble fraction) was determined (Table 5). In this way free phenolic compounds would be recovered mainly in the TCA-soluble fraction, whereas protein-bound phenolic compounds would

Table 4
Antioxidant capacity of the filmogenic dispersions and the water-soluble fraction of the resulting sunflower protein films, evaluated by the ABTS, FRAP and PCL assays.

Sample		ABTS (mg/ml or g)	FRAP (mmol/ml or g)	PCL-ACW (μmol/ml or mg)	PCL-ACL (μmol/ml or mg)
Filmogenic dispersion	**SFPC**	0.18 ± 0.01^{a}	3.99 ± 0.12^{a}	22.60 ± 0.30^{a}	15.80 ± 0.30^{a}
	SFPC + IP	0.12 ± 0.01^{b}	2.55 ± 0.05^{b}	0.90 ± 0.30^{b}	4.10 ± 0.30^{b}
Protein film	**SFPC**	65.26 ± 2.16^{c}	545.80 ± 18.60^{c}	858.60 ± 10.10^{c}	160.10 ± 1.30^{c}
	SFPC + IP	33.24 ± 1.13^{d}	200.10 ± 14.17^{d}	34.51 ± 2.10^{d}	149.80 ± 1.00^{d}

Reported values for each protein product are means ± standard deviation. Different letters (a, b, c, d) in the same column indicate significant differences ($p < 0.05$) according to Tukey's test.

remain mostly in the TCA-insoluble fraction. Phenolic compounds from **SFPC** samples (both filmogenic dispersion and film aqueous extract) were found mostly in the TCA-soluble fraction, indicating that they predominate in the free form as could be expected from the nature and composition of the starting sunflower protein concentrate. Moreover, in the **SFPC** fraction the difference in the percent content of phenolic compounds between the soluble and insoluble fractions of the filmogenic dispersion diminished markedly (from 37% to 18%) when the value in the respective film was considered. This indicates that during **SFPC** film formation (possibly during the drying step) some free phenolic compounds in the filmogenic dispersion have a greater chance to interact with proteins, thus increasing the percentage of protein-bound phenolic compounds. Furthermore, the involvement of the residual water-soluble carbohydrate content in **SFPC** on interactions with both phenolic compounds and proteins should not be disregarded (McManus et al., 1985; Peinado, López de Lerma, & Peinado, 2010). In contrast, no increase in the proportion of protein-bound phenolic compounds was observed in association with film formation in **SFPC + IP**, probably due to the lower proportion of free phenolic compounds content found in the raw material.

Therefore, the lower antioxidant capacity of **SFPC + IP** films as compared to **SFPC** films can be attributed not only to the lower absolute content of total phenolic compounds, but also to a greater degree of protein-phenolic interaction in the starting protein concentrate. These results agree with those reported by Gómez-Estaca, Bravo, Gómez-Guillén, Alemán, and Montero (2009), whose found that films obtained from bovine gelatin films added with rosemary or oregano extracts exhibited a higher antioxidant capacity (as measured by FRAP and ABTS assays) than films made from fish gelatin. The greater tendency of fish gelatin to interact with phenolic compounds was considered a determinant factor for the lower antioxidant capacity of the respective films.

3.5. Antimicrobial properties

In spite of the antimicrobial activity of chlorogenic and caffeic acids reported in the literature (Bowles & Miller, 1994; Gómez-Estaca et al., 2010; Klančnik et al., 2009), the phenolic compounds naturally present in the sunflower protein concentrates studied here, considering either the filmogenic dispersions or the resulting protein films, showed no antimicrobial activity against the studied microorganisms (data not shown). The antimicrobial activity of phenolic compounds in relation with their chemical structure has been reviewed by Puupponen-Pimiä, Nohynek, Alakomi, and Oksman-Caldentey (2005). The activity appears to be related to damage to the cytoplasmic membrane and other structures, reduction of the pH gradient across the cytoplasmic membrane; permeabilization (generally by hydrophobic compounds able to disintegrate the lipopolysaccharide layer of the outer membrane of Gram-negative organisms); inhibition of extracellular microbial enzymes; inhibition of oxygen consumption and disruption of the membrane-associated respiratory chain, etc. In this connection, the differential sensitivity of Gram-positive (more sensitive) and Gram-negative bacteria to phenolic compounds was due to the different structure of their respective cell walls (Puupponen-Pimiä et al., 2005; von Staszweski et al., 2011).

Table 5
Percentage of phenolic compounds, with respect to total content, that precipitate (insoluble fraction) or not (soluble fraction) with TCA (10% w/v) in the filmogenic dispersions and in the film aqueous extracts of sunflower protein films.

Sample		TCA-soluble fraction (%)	TCA-insoluble fraction (%)
Filmogenic dispersion	**SFPC**	67.3 ± 0.2^{a}	30.7 ± 2.9^{a}
	SFPC + IP	34.1 ± 2.0^{b}	67.5 ± 0.4^{b}
Film aqueous extract	**SFPC**	57.8 ± 1.7^{c}	39.8 ± 1.6^{c}
	SFPC + IP	41.1 ± 2.1^{d}	61.7 ± 0.4^{d}

Reported values for each protein product are means ± standard deviation. Different letters (a, b, c, d) in the same column indicate significant differences ($p < 0.05$) according to Tukey's test.

In our study, the lack of antimicrobial activity may be attributed to interactions between phenolic compounds and proteins, as discussed above. In a previous study, the antimicrobial effect of the polyphenols (from green tea infusions) in the presence of whey proteins increased with the reduction of whey protein concentration (von Staszweski et al., 2011). These authors found that the effect of green tea polyphenols is concentration dependent, and the food matrix can decrease the antimicrobial activity of these compounds.

The pH is another factor that highly influences the activity of phenolic compounds. von Staszewski et al. (2011) reported that the antimicrobial effectiveness of polyphenols (from Argentinean green tea) did not vary significantly in a pH range of 4.0–7.0. Some phenolic acids (chlorogenic, caffeic and gallic acids) are not stable at high pH, and the spectral transformations that depend on time and pH are not reversible (Friedman & Jürgens, 2000). The inhibition of *L. monocytogenes* growth by phenolic acids (cinamic, p-coumaric, ferulic and caffeic acids) increased with decreasing pH (Kouassi & Shelef, 1998; Wen, Delaquis, Stanich, & Toivonen, 2003), although the antilisterial effect of chlorogenic acid was greater at pH 6.5 than 5.5 (Wen et al., 2003). These authors found that the minimal inhibitory concentration of chlorogenic acid (p*K* 3.4 and p*K* 8.5) against *L. monocytogenes* was 0.40% at pH 8.5 and >1% at pH 6.26, which indicates that changes in the ionization state and proportion of undissociated molecules modify the activity of the acid. In the present study, the pH of the filmogenic dispersions was around 11; under these conditions the main phenolic acids (chlorogenic and caffeic acids) present in both studied protein concentrates were not expected to be in the undissociated state and therefore, could not exert an antimicrobial effect.

In our work, the phenolic compounds were not evaluated as isolated species but as compounds naturally present in sunflower protein concentrates. As previously mentioned, the protein-phenolic compounds interactions and the influence of pH could explain, as least in part, the lack of antimicrobial activity. Further studies testing filmogenic dispersions at different pH values in order to facilitate the activity of phenolic compounds, and the inclusion of different concentrations of microorganisms are

necessary to evaluate the antimicrobial activity of sunflower phenolic compounds.

4. Conclusions

Phenolic compounds naturally present in the sunflower protein concentrates studied conferred antioxidant properties to the resulting protein films, but showed no antimicrobial activity. The antioxidant activity exhibited by filmogenic dispersions and films depended on the content of phenolic compounds and on their free or protein-bound state. Since the antioxidant activity of phenolic compounds was preserved during the protein concentration process (inherent to film formation), these protein matrices could be envisioned as useful systems for protecting such bioactive compounds. Finally, taking into account that films made from sunflower proteins present not only antioxidant properties but also a strong color that endows them with light barrier properties, together with physicochemical properties adequate for packaging, the use of these materials can be proposed for protecting products that are susceptible to oxidation. Further studies are needed in order to elucidate the contribution of residual carbohydrates in sunflower protein concentrates to physical and antioxidant properties of resulting films.

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