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Influence of peptides–phenolics interaction on the antioxidant profile of protein hydrolysates from *Brassica napus*

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ABSTRACT

The role of the peptides–phenolic compounds (PC) interaction on the antioxidant capacity profile (ACP) of protein hydrolysates from rapeseed (*Brassica napus*) was studied in 36 hydrolysates obtained from a PC-rich and PC-reduced protein substrate. The latent profile analysis (LPA), with data of seven *in vitro* methods and one assay for cellular antioxidant activity (CAA), allowed identifying five distinctive groups of hydrolysates, each one with distinctive ACP. The interaction of peptides with naturally present PC diminished *in vitro* antioxidant activity in comparison with their PC-reduced counterparts. However, CAA increased when peptides–PC interaction occurred. The profile with the highest average CAA (62.41±1.48%), shown by hydrolysates obtained by using alcalase, shared typical values of Cu^{2+}-catalysed β-carotene oxidation (62.41±0.43%), β-carotene bleaching inhibition (91.75±0.22%) and Cu^{2+}-chelating activity (74.53±0.58%). The possibilities for a sample to exhibit ACP with higher CAA increased with each unit of positively charged amino acids, according to multinomial logistic regression analysis.

1. Introduction

Rapeseed (*Brassica napus*) is a cruciferous oilseed plant economically important for oil production worldwide. The by-product generated once the oil has been removed is known as rapeseed meal (RM). This meal is a complex matrix composed of storage proteins, and insoluble fibre but it also contains phenolic compounds, pythic acid, glucosinolates and chlorophylls among others components. The main phenolic substance present in rapeseed is 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid). Sinapic acid occurs in rapeseed in free acid form; however the most significant of the phenolic compounds in rapeseed is sinapine, the choline ester of sinapic acid (Khattab, Goldberg, Lin, & Thiyam, 2010; Thiyam, Stöckmann, Zum Felde, & Schwarz, 2006). Sinapic acid in rapeseed also exists as, glucopyranosyl sinapate (Amarowicz & Shahidi, 1994). Most of the phenolic compounds remain in the meal when the oil is pressed from the seeds and may complex with proteins either reversibly by a hydrogen-bonding mechanism or irreversibly by oxidation to quinones, which then combine with reactive groups of protein molecules causing poor digestibility and undesirable coloration and taste and therefore they have limited rapeseed proteins use in human foods (Aider & Barbana, 2011; Tan, Mailer, Blanchard, & Agboola, 2011a). At the same time, beneficial bioactivities as antihypertensive, antioxidant or anticancer properties have been reported for phenolics, protein hydrolysates and bioactive peptides from rapeseed (Cumby, Zhong, Naczk, & Shahidi, 2008; He, Girgih, Malomo, Ju, & Aluko, 2013; Khattab et al., 2010).

Among potential bioactive components embedded into RM, some sequences of rapeseed proteins with biological activity, known as bioactive peptides, can be released by enzymatic hydrolysis from their parent proteins. These released fragments (pure or as a mixture) may be able to exert antioxidant activity. Recently, studies have proposed that hydrolysis of rapeseed proteins is a suitable alternative for RM exploitation and a potential low cost source for bioactive peptides production (Aachary & Thiyam, 2011; He et al., 2013; Mäkinen, Johannson, Vegarud Gerd, Pihlava, & Pihlanto, 2012). However, important difficulties arise when preparing antioxidant hydrolysates from this source, as well as from other common vegetal sources, such as those related to protein separation, extraction and purification from other components before the hydrolysis process. The separation of proteins from phenolic compounds is complicated by their complex-forming mechanisms. Therefore additional steps have been added to protein isolation procedures in order to remove components that may establish an interaction with proteins since the former might

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reduce enzyme activity, sensory attributes or activity of peptides (Flurkey et al., 2008; Tan, Mailer, Blanchard, & Agboola, 2011b). Nevertheless, every additional purification step means an increase of production cost. Hence, protein concentrates with less than 90% of protein have been used as starting materials for producing hydrolysates. Consequently, it is important to evaluate the influence of remaining components on the antioxidant activity of peptides. This has been mainly addressed for PC, whose antioxidant characteristics as well as their ability to remain associated to proteins have been reported (Papadopoulou & Frazier, 2004). These particular associations result in complex mixtures of bioactive, non-bioactive peptides as well as phenolic-associated peptides (Bourseau et al., 2009).

Besides the heterogeneity of peptide mixtures, there are other challenges for producing antioxidant hydrolysates, for instance, the difficulty to compare and/or provide a scale of antioxidant activity due to the lack of a single universal method to assess it (Niki, 2010). Therefore, this work on rapeseed protein hydrolysates (RPHs) was aimed to investigate the influence of the peptides–phenolics interaction on the antioxidant capacity profile (ACP) defined as the exclusive combination of values from antioxidant capacity assays determined by using latent profile analysis (LPA), a multivariate statistics technique that gathers observations according to the value that each observation takes in the independent variable axis (antioxidant capabilities assays). Each of the mutually exclusive groups represents a category of a latent variable (the antioxidant capacity profile) which cannot be directly observed.

Data from six observations of seven *in vitro* assays and the results from the cellular antioxidant assay of 36 protein hydrolysates obtained by hydrolysis of two different substrates: rapeseed protein concentrate (RPC) and rapeseed protein isolate (RPI) were analysed. Then, multinomial logistic regression was applied in order to identify chemical or physical characteristics of hydrolysates related to the probability of an observation belonging to one specific antioxidant profile. These results would provide meaningful information for supporting the value-adding use of the rapeseed by-product.

2. Material and methods

2.1. Material and reagents

Rapeseed (*B. napus*) (00) var. Hyola 401 grown in Estado de México, México during Spring-Summer cycle, alcalase 2.4L (EC 3.4.21.62, 2.4 Anson Units g^{-1}), flavourzyme (EC 3.4.11.1, 500 LAPU g^{-1}), pepsin A (E.C.3.4.23.1, PP-77163, 800–2500 units/mg protein, from hog stomach), pancreatin (P-1750, 4XUSP, from porcine pancreas), TNBS (2,4,6-trinitrobenzenesulphonic acid), DPPH (2,2 diphenyl-1 picrylhydrazyl), ABTS [2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate)], Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), Tween 20, $K_3Fe(CN)_6$, $FeCl_3$, $FeCl_2$, BHT (butylated hydroxytoluene tetrahydrate), ferrozine, pyrocatechol violet, linoleic acid, β-carotene, sinapic acid, EDTA (ethylenediaminetetraacetic acid), TBH (*tert*-butyl hydroperoxide), D,L-α-aminobutyric acid, trypan blue and DCFH-DA (20,70-dichlorofluorescein diacetate) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HBSS (Hank's Balanced Salt Solution), DMEM (Dulbecco's Modified Eagle's Medium), foetal bovine serum and antibiotics for cell culture were from GIBCO (Barcelona, Spain). All other chemicals were of analytical grade provided by JT Baker (Phillipsburg, NJ, USA), Merck (Darmstadt, Germany).

2.2. Preparation of defatted flours

Rapeseed starting materials were prepared from the de-oiling residue obtained by pressing of *B. napus* var. Hyola seed. Seed was first extracted with hexane in a Soxhlet system for 9 h in order to remove the fat residue, afterwards, it was ground to a powder which was passed through a 0.2 mm-mesh sieve. Additional steps were used to remove polyphenols and others compounds from the flour designated to obtain the rapeseed protein isolate, according to Vioque, Alaiz, and Girón-Calle (2012) with slight modifications, by performing two extractions with 75% acetone at 4 °C for 1 h followed by two extractions with methanol absolute under same time and conditions. After centrifugation at 7000 g the precipitate was collected, nitrogen-dried and stored at −20 °C.

2.3. Preparation of rapeseed protein concentrate (RPC) and isolate (RPI)

Two protein substrates for enzymatic hydrolysis were prepared from defatted and polyphenols-reduced flours, respectively named rapeseed protein concentrate (RPC) and rapeseed protein isolate (RPI) according to the method of Sánchez-Vioque, Clemente, Vioque, Bautista, and Millán (1999). Briefly, the corresponding flour was suspended in water (1:10 (w/v)), then 0.05% (w/v) of Na_2SO_3 was added to prevent oxidation of phenolics, adjusted to pH 12 for protein solubilisation and maintained constant by the addition of 1 M NaOH under stirring for 1 h. The slurry was centrifuged at 10,000 g for 30 min; the pellet obtained was extracted once more with half of volume under the same conditions. The combined supernatants were first adjusted to isoelectric point at pH 5, the resulting precipitate was recovered by centrifugation as described above and the supernatant was adjusted to a second isoelectric point at 3.5 and then treated in the same way. The precipitates were mixed, washed with water, adjusted to pH 7 and freeze-dried. Protein content was determined as further indicated in Section 2.6.3.

2.4. Enzymatic hydrolysis

RPC and RPI were hydrolysed with two different combinations of enzymes: (**1**) alcalase–flavourzyme (AF) and (**2**) pepsin–pancreatin (PP). The first enzyme of each combination was added at time 0 while the second one 120 min later. Hydrolysis parameters for each system were as follows: RPC or RPI concentration, 4% (w/v) in both systems; temperature for alcalase–flavourzyme, 50±0.2 °C; temperature for pepsin–pancreatin system, 37±0.2 °C; enzyme/substrate ratios and pH were 0.3 AU g^{-1} and pH 8 for alcalase, 50 LAPU g^{-1} and pH 7 for flavourzyme, 1/100 (w/w) and pH 2 for pepsin, 1/100 (w/w) and pH 8 for pancreatin. Aliquots were taken at 0, 30, 60, 90, 120, 150, 180, 210 and 240 min. Proteases were inactivated by heating at 80 °C for 5 min. Hydrolysates were clarified by isoelectric precipitation of non-hydrolysed protein and centrifugation at 10,000g in order to remove the residual enzyme and insoluble substrate, afterwards they were freeze-dried and stored at −20 °C.

2.5. Preparation of polyphenols extract

The rapeseed polyphenols extract (RPE) used for antioxidant determinations was prepared according to Khattab et al. (2010) with slight modifications, defatted flour (10% w/v) was extracted four times with 70% methanol for 1 h at 4 °C. The extract was recovered by centrifugation at 7500 g for 15 min, concentrated under vacuum evaporation and stored in the dark at −20 °C.

2.6. Characterisation of hydrolysates

2.6.1. Degree of Hydrolysis

The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS according to Adler-Nissen (1979). Total number of amino groups was determined in a sample, 100% hydrolysed by treatment with 6 M HCl at 110 °C for 24 h, of each hydrolysate and calculated as follows:

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} \times 100 \quad (1)$$

where h = number of hydrolysed peptide bonds, and h_{tot} = number of peptide bonds per protein equivalent.

2.6.2. Determination of total phenolic content

Total content of polyphenols in the hydrolysates was determined by using the Folin–Ciocalteu reagent as described in Singleton, Orthofer, and Lamuela-Raventós (1999). Using sinapic acid as standard, total phenolics content (TPC) results are given as mg sinapic acid equivalents/g (mg SAE/g).

2.6.3. Amino acids composition analysis and free amino acid content

The amino acids composition of rapeseed flours, concentrate, isolate and hydrolysates was determined by RP-HPLC after sample hydrolysis (40 μg), with 6 M HCl (1 mL) at 110 °C for 24 h in tubes sealed under nitrogen, and amino acids derivatisation with diethyl ethoxymethylenemalonate (by using D,L-α-aminobutyric acid as an internal standard) according to Alaiz, Navarro, Girón, and Vioque (1992). Tryptophan content was analysed after alkaline hydrolysis according to Yust et al. (2004) with modifications. Briefly, samples were hydrolysed in 3 mL of 4 M NaOH at 100 °C for 4 h. Hydrolysates were neutralised, nitrogen dried and resuspended in 1 mL 1 M sodium borate buffer (pH 9).

The HPLC (Beckman-Coulter, Brea, CA, USA) was equipped with a 300 mm × 3.9 mm (internal diameter), 4-μm, Novapack C18 reversed-phase column (Waters, Milford. MA, USA). A binary gradient was used for elution with a flow of 0.9 mL/min. The solvents used were A: sodium acetate (25 mM) containing sodium azide (0.02% (w/v)), pH 6.0, and B CH_3CN. Elution was as follows: time, 0–3 min, linear gradient from A/B (91/9) to A/B (86/14); 3–13 min, elution with A/B (86/14); 13–30 min, linear gradient from A/B (86:14) to A/B (69/31); and 30–35 min, elution with A/B (69/31). The column was maintained at 18 °C. Detection was carried out at 280 nm. Free amino acid content was determined for non-hydrolysed samples as described above.

2.6.4. Determination of average molecular weight (AMW) distributions

The molecular mass distribution profile was determined by gel filtration chromatography carried out in an AKTA-purifier FPLC system equipped with a Superdex Peptide, 10/300 GL column (GE Healthcare, Piscataway, NJ, USA). Injection volume and protein concentration were 500 μL and 0.2 mg/mL, respectively. The eluent was 0.75 M ammonium bicarbonate and elution was monitored at 215 nm. Molecular masses were determined by using blue dextran (2000 kDa), cytochrome C (12.5 kDa), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da) as molecular weight standards (Amersham Pharmacia LKB Biotechnology, Uppsala, Sweden).

2.6.5. High performance liquid chromatography (HPLC) peptide profiles

HPLC peptide profiles were acquired by injection of 20 μL of hydrolysates with protein concentration of 5 μg/μL in a chromatographic system (Beckman-Coulter, Brea, CA, USA) equipped with a Discovery BIO Wide Pore C18 column, 25 cm × 4.6 mm, 5 μm (Supelco, Bellefonte, PA, Pennsylvania, USA.) and UV–vis detector. A binary elution gradient, with solvent A: water containing 0.1% (v/v) trifluoroacetic acid and B: acetonitrile containing 0.1% (v/v) trifluoroacetic acid, was used with an initial composition of A/B (100/0) to A/B (70/30) for 60 min at a flow rate of 1 mL/min, 25 °C, and detection at 215 nm. Data acquisition and processing were performed by using the 32 Karat 7.0 software (Beckman-Coulter, Brea, CA, USA).

2.7. In vitro measurement of antioxidant properties

2.7.1. Scavenging of DPPH radical

The DPPH radical scavenging activity was determined according to Shimada, Fujikawa, Yahara, and Nakamura (1992) with slight modifications. Final concentrations ranged 0.167–5.0 μg/μL and 0.001–0.06 μg/μL for aqueous solutions of RPHs and RPE or standard, respectively. The absorbance was measured after 30 min at 517 nm by using a Thermo Lab Systems Multiskan Spectrum microplate spectrophotometer (Vantaa, Finland). The radical scavenging capacity was calculated by the following equation:

$$\text{DPPH Radical Scavenging Activity } (\%) = \frac{A_b - A_s}{A_b} \times 100 \quad (2)$$

where A_b and A_s are the absorbance of the blank and sample, respectively. The effective concentration that scavenged 50% of the free radicals (EC_{50}, DPPH) was calculated for samples by non-linear regression from a plot of% of DPPH activity *vs.* sample concentration (μg/μL).

Distilled water, methanol and sinapic acid (SA) were used as blank and standard, respectively, in all antioxidant activity assays.

2.7.2. Scavenging of $ABTS^{\cdot+}$

The ABTS radical cation ($ABTS^{\cdot+}$) decolouration assay was performed according to Re et al. (1999); briefly, $ABTS^{\cdot+}$ was produced by reaction of ABTS (7 mM in water) with $K_2S_2O_8$ (2.45 mM final concentration) in the dark at room temperature for more than 12 h. Prior to the assay, the solution was diluted in ethanol and equilibrated at room temperature in order to obtain an absorbance of 0.70±0.02 at 734 nm. Samples (20 μL) were incubated for 6 min with 180 μL $ABTS^{\cdot+}$ solution to final concentrations ranged 0.001–0.1 μg/mL and 0.0005–0.04 μg/mL for aqueous solutions of RPHs and RPE or SA, respectively. Absorbance was measured at 734 nm, and percentage of $ABTS^{\cdot+}$ scavenged was calculated by using the equation:

$$\text{Percentage } ABTS^{\cdot+} \text{ scavenged } (\%) = \frac{A_i - A_f}{A_i} \times 100 \quad (3)$$

where A_i and A_f are initial and final absorbance of the sample, respectively. Percentages were interpolated on a trolox standard curve (0.0–0.8 mM) and expressed as mM Trolox equivalent antioxidant capacity (TEAC)/g for RPH comparison. The effective concentration that scavenged 50% of the free radicals (EC_{50}, $ABTS^{\cdot+}$) was calculated for selected hydrolysates and standards by non-linear regression from a plot of percentage $ABTS^{\cdot+}$ scavenged *vs.* sample concentration (μg/μL).

2.7.3. β-Carotene bleaching method

Antioxidant activity was estimated by determination of protection against the peroxidative decomposition of β-carotene (bleaching) in the presence of linoleic acid and the samples as described by Pastor-Cavada, Juan, Pastor, Alaiz, and Vioque (2009) with modifications. A mixture of β-carotene (1 mL, 2 mg/mL in chloroform), linoleic acid (20 mg) and Tween 20 (200 μg) was vortexed and flushed with nitrogen in order to eliminate chloroform. After addition of oxygen-sparged distilled water (20 mL), the mixture was diluted to a clear solution of Abs 1.2±0.02 at 450 nm. The sample solutions (20 μL) and β-carotene assay solution (200 μL) were

added to reach final concentrations about 0.01–0.9 μg/μL and 0.001–0.04 μg/mL, for aqueous solutions of RPHs RPH and RPE or SA, respectively, and incubated at 50 °C. Absorbance (450 nm) was read at 10 min intervals during 1 h. Data on the inhibition of β-carotene bleaching by the samples were processed as follow:

$$\text{Antioxidant activity } (\%) = \frac{DR_b - DR_s}{DR_b} \quad (4)$$

where DR_b and DR_s are the blank and sample degradation rates, respectively, calculated as:

$$DR = \frac{\left(\text{Ln}\frac{\text{Abs at 0 min}}{\text{Abs at 60 min}}\right)}{60} \quad (5)$$

The effective concentration that inhibits 50% of β-carotene bleaching (EC_{50}) was calculated for samples by non-linear regression from a plot of% of antioxidant activity *vs*. sample concentration (μg/μL).

2.7.4. Copper catalysed β-carotene oxidation

The assay of β-carotene oxidation was carried out as described in the literature after some modifications (Pedroche et al., 2006). A solution of β-carotene was prepared by dissolving 4 mg in 1 mL of chloroform. After addition of 1 mL of Tween 20, chloroform was evaporated under nitrogen. Aliquots from this solution were dissolved in 100 mM pH 7.4 phosphate buffer. A 10 mM solution of $CuSO_4$ was prepared and 2 mL were added to each well. Final concentrations in the assay mixture (200 mL) were: 119 mM β-carotene, 0.1 mM Cu^{2+} and concentrations about 0.01–0.9 μg/μL and 0.001–0.9 μg/μL for RPH and RPE, respectively. The degradation of β-carotene was monitored by recording the decrease in absorbance at 470 nm using a ThermoLab Systems Multiskan Spectrum microplate spectrophotometer (Vantaa, Finland). Calculations were as described in Section 2.7.3.

2.7.5. Reducing power

The reducing power was determined according to Oyaizu (1986) after some modifications. Hydrolysates (20 μL) at concentrations comprised between 0.1–5.0 μg/μL and 0.01–0.4 μg/μL, for RPH, RPE or SA, respectively, were added to 50 μL of 0.2 M phosphate buffer (pH 6.6) and 50 μL of 1% $K_3Fe(CN)_6$ incubated at 50 °C for 20 min. Plates were incubated for another 10 min at 50 °C after addition of 50 μL of 10% trichloroacetic acid and 10 μL of 0.1% $FeCl_3$, and absorbance was read at 700 nm.

The effective concentration defined as the peptide concentration at which the absorbance at 700 nm was 0.5 for reducing power (EC50) was calculated for samples by non-linear regression from a plot of absorbance at 700 nm *vs*. sample concentration (μg/μL).

2.7.6. Iron chelating activity

Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}–ferrozine coloured complex at 562 nm (Carter, 1971). Samples (100 μg) were mixed with 250 μL of 100 mM sodium acetate buffer pH 4.9, and 30 μL of $FeCl_2$ (0.01%, w/v). Ferrozine (12.5 μL, 40 mM) was added after incubation for 30 min at room temperature. EDTA was used as a positive control. Binding of Fe^{2+} ions to ferrozine generates a coloured complex that was measured at 562 nm by using a microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA).

Iron chelating activity was calculated by using the following equation:

$$Fe^{2+} \text{ chelating activity} = \frac{A_b - A_s}{Ac} \times 100 \quad (6)$$

where A_b and A_s are the blank and sample absorbances.

2.7.7. Copper chelating activity

Cu^{2+} chelating activity was determined according to Saiga, Tanabe, and Nishimura (2003). 50 mM sodium acetate buffer, pH 6 (290 μL,), 6 μL of 4 mM pyrocatechol violet prepared in the same buffer, and $CuSO_4{\cdot}H_2O$ (1 μg) were added to the hydrolysates (100 μg) with concentrations of 0.02–0.6 μg/μL and 0.001–0.5 μg/μL, for RPH and RPE or SA, respectively. Absorbance was measured at 632 nm. Calculations were as described in Section 2.7.6.

The effective concentration (EC_{50}) was calculated for each chelating activity by non-linear regression from a plot of percentage activity *vs*. sample concentration (μg/μL).

2.8. Cellular antioxidant activity

2.8.1. Cell culture

Caco-2 cells were obtained from the European Cell Culture Collection (ECACC; number 86010202, Salisbury, UK) and cultured under standard cell culturing conditions (5% CO_2 at 37 °C) in DMEM medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin and cells were sub-cultured twice a week. Trypan blue dye was used to asses cellular viability.

2.8.2. DCFH-DA assay

Intracellular formation of reactive oxygen species (ROS) was assessed in Caco-2 cell as described by Wang and Joseph (1999) by measuring the *tert*-butyl hydroperoxide (TBH)-induced production of free radicals using the oxidation-sensitive dye DCFH-DA as the substrate.

Confluent cells were seeded in 96 well microplates (100 μL, 2×10^5 cells/well) one week before the assays. Growth medium was removed and cells were washed with HBSS before the addition of samples. Cells were then treated with 90 μL of samples with different concentrations about 1.0–9.0 and 0.1–3.0 μg/μL, for RPH and RPE or SA, respectively, in HBSS and pre-incubated for 24 h at 37 °C allowing for sample absorption. After incubation, the non-absorbed sample and growth medium were removed and cells were washed with fresh HBSS medium. Volumes of 90 μL of 25 μM DCFH-DA in HBSS were added and incubated for 1 h in the dark. Finally, plates were loaded with 10 μL of 250 μM THB solution. The formation of 20,70-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of ROS was read after every 15 min, at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 555 nm, by using a Fluoroskan Ascent plate-reader (Thermo Scientific, MA, USA). Each plate included one positive control (cells treated with DCFH-DA and the free radical generator TBH) and one negative control (cells treated only with DCFH-DA). The negative control was included in order to prevent overestimation of free radicals due to photo oxidation of DCFH. The lower the fluorescence emission in the presence of hydrolysates, the lower the free radical concentration due to the antioxidant activity of hydrolysates. Antioxidant activity was calculated as follows, as a function of emitted fluorescence (% IF):

$$\%\,\text{IF} = \frac{\text{Fluorescence at time 30 min} - \text{Fluorescence at time 0 min}}{\text{Fluorescence at time 0 min}} \times 100 \quad (7)$$

Dose-dependent effects of treatment groups were plotted and compared to that of fluorescence intensity from control and blank groups. The effective concentration of sample that reduced fluorescence by 50% (EC_{50}) was calculated from a non-linear regression plot of% IF *vs*. sample concentration (μg/μL).

2.9. Statistical analysis

Results were expressed as mean ± standard error ($n = 6$). The means were analysed by Shapiro–Wilk test for normality assessment. The means comparison was done by One-way ANOVA test, and differences among means were determined by using Fisher's least significant difference for normally distributed populations, whereas Kruskal–Wallis test was used when populations failed the normality test. This was performed by using the software Statgraphics Centurion, Version 16.2, (Statpoint Technologies, Inc., Rockville MD, USA).

2.9.1. Latent profile analysis

Clustering of data on antioxidant capacity of RPHs was carried out by Latent Profile Analysis (LPA) based on the hypothesis that heterogeneous population might be segmented into homogeneous mutually-exclusive subgroups according to a distinctive latent variable (unobservable) corresponding to the combination of antioxidant capacity results (profile). In order to identify the number of profiles between RPHs, a latent profile model was constructed and selected with Mplus 6.11 software (Asparouhov & Muthén, 2012), model fitting was performed and selection parameters were statistical hypothesis test, entropy, log- likelihood, Bayesian Information Criteria (BIC) and Akaike information criterion (AIC).

2.9.2. Multinomial logistic regression

In order to test theoretical approaches that relate polyphenols content, degree of hydrolysis (DH), and amino acidic composition (AA) with groups established by LPA, multinomial logistic regression models were tested with the purpose of finding physical or chemical characteristics influencing each sample for belonging to certain LPA category.

3. Results and discussion

3.1. Preparation of rapeseed protein hydrolysates (RPHs) from rapeseed protein concentrate (RPC) and rapeseed protein isolate (RPI)

Different treatments based on protein extraction were applied with the aim of allowing obtaining protein substrates for enzymatic hydrolysis from two different rapeseed flours. Once compounds such as fibre were removed from rapeseed defatted flour, and the soluble protein was extracted a protein rich product (87.82±0.12%) named rapeseed protein concentrate (RPC) was obtained. The increase of 2.44 times in protein concentration in RPC also lead a proportional increase of minor non-protein compounds, phenolic compounds included, that may remained bound to proteins at isoelectric pH (Papadopoulou & Frazier, 2004; Xu & Diosady, 2000, 2002). In this study colorimetric analysis for total extractable phenolic compounds, which beside sinapic acid and its derivatives also include proanthocyanidins and extractable tannins indicated TPC between 0.50±0.01 and 0.85±0.01 g SAE/100 g for the protein hydrolysates obtained from RPC (Table 2). This is about 21–25% of the extractable phenolic compounds originally present in the flour (1.3662±0.01 g/100 g). Xu and Diosady (2000) reported that lowering the pH of the extracts from 12.0 to 3.5 substantially reduced the ionically bonded fraction of phenolic acids, mainly sinapine, in agreement with our results only a proportion of about 20% of the extractable phenolic compounds originally present in the flour was found to precipitated with proteins (Xu & Diosady, 2000, 2002).

On the other hand a polyphenols-reduced protein substrate, named rapeseed protein isolate (RPI), was obtained from the rapeseed defatted flour extracted with solvents for phenolic compounds reduction; these previous purification steps allowed the production of a material with 97.9±0.08% protein content (RPI). Decrease of polyphenols from starting material was possible since several reversible mechanisms are involved in the interaction of phenolic compounds and rapeseed proteins into some complexes; among these, ionic bonding has been reported as predominant (accounting for 30% of extractable phenolics) but other reversible interactions, for instance non-covalent forces such as hydrogen bonding, hydrophobic bonding and van der Waals forces, usually are involved as well (Xu & Diosady, 2000).

3.2. Influence of proteins–phenolic compounds interaction on hydrolysis process

The influence of TPC at different points of the hydrolysis processes of RPC and RPI by AF or PP sequential enzymatic treatments are presented in Table 1. Significant differences in TPC concentrations ($p = 0.05$) were found for all hydrolysates obtained from RPC in comparison with their analogous samples obtained from RPI. RPHs prepared from RPC had between 3 and 4.5-fold higher concentrations of TPC than those found for hydrolysates obtained from RPI.

The degree of hydrolysis (DH) was 1.48 and 6.14% higher for phenolic-reduced substrate at the end of PP and AF enzymatic treatments, respectively. Important differences were found in RP-HPLC peptide profiles, molecular weight distributions, total amino acid and free amino acid contents (Fig. 1 and Table 2) with the occurrence of most changes in the fragmentation pattern of both substrates once the corresponding second enzyme was added. In general, more fragmentation was achieved with AF since the broader specificity of alcalase and exopeptidase activity of flavourzyme led to more excision sites in comparison to pepsin which is an endoprotease efficient for cleaving bonds involving the aromatic amino acids phenylalanine, tryptophan and tyrosine but this specificity could have limited the rate of canola protein hydrolysis and hence decreased the amount of hydrolysis excision sites. Pancreatin as a mixture of endo- and exoproteases was probably responsible for the increase in the degree of fragmentation and free amino acids release obtained (Doucet, Otter, Gauthier, & Foegeding, 2003; Torres-Fuentes, Alaiz, & Vioque, 2011). More peptides were released when phenolic-reduced substrate (RPI) was used according to RP-HPLC peptide profiles and in agreement with the higher DHs, although higher amount of free amino acids which may diminish antioxidant properties (Elias, Kellerby, & Decker, 2008; Ozdal, Capanoglu, & Altay, 2013) were found as well, mostly hydrophobic 49.7 and 48.9% of total free amino acid content for AF and PP, respectively.

The differences observed in AMW distributions of peptide mixtures from RPC and RPI are presented in Table 2, hydrolysis with AF of both substrates led to a greater fraction of fragments below 11.8 kDa in comparison to PP. In the AMW distribution of extensive hydrolysates obtained from RPI, an increase of high MW peptides was observed and this may be due to low peptide association into aggregates of elevated molecular weight since high amount of hydrophobic peptides were found in extensive hydrolysates according to peptide profile figures (Fig. 1).

The antioxidant potential of protein hydrolysates depends on number of fragments, length, amino acid composition, complexes formed with other compounds including polyphenols, secondary structure and disruption of tertiary structure of parent proteins by enzymatic hydrolysis that results in increasing the solvent accessibility and exposition of oxidation-labile amino acid residues (Elias et al., 2008; Ozdal et al., 2013). Therefore, major differences of hydrolysates prepared from both protein substrates under different hydrolysis conditions and enzyme combination might be related to antioxidant capacity profiles (ACP). Hence, these results may serve to estimate differences in fragmentation of both

Table 1
Total phenolic content (TPC) and degree of hydrolysis of rapeseed protein hydrolysates obtained from rapeseed protein concentrate (RPC) and rapeseed protein isolate (RPI).

Enzymes	Rapeseed protein concentrate (RPC)						Rapeseed protein isolate (RPI)					
	Alcalase–flavourzyme			Pepsin–pancreatin			Alcalase–flavourzyme			Pepsin–pancreatin		
Hydrolysis time (min)	Sample code	TPC (g SAE/100 g)	DH (%)	Sample code	TPC (g SAE/100 g)	DH (%)	Sample code	TPC (g SAE/100 g)	DH (%)	Sample code	TPC (g SAE/100 g)	DH (%)
0	PCAF1	0.71 ± 0.01^{i}	1.65 ± 0.14^{a}	PCPP1	0.85 ± 0.01^{g}	0.71 ± 0.10^{a}	AF1	$0.16\pm0.01^{e,f,g}$	1.24 ± 0.11^{a}	PP1	$0.16\pm0.00^{f,g}$	1.35 ± 0.14^{a}
30	PCAF2	$0.65\pm0.01^{e,f,g}$	23.11 ± 1.23^{b}	PCPP2	0.51 ± 0.00^{b}	4.42 ± 0.18^{b}	AF2	$0.15\pm0.01^{a,b,c,f}$	27.57 ± 0.28^{b}	PP2	0.15 ± 0.00^{b}	4.94 ± 0.93^{b}
60	PCAF3	0.57 ± 0.01^{a}	29.89 ± 1.34^{c}	PCPP3	$0.50\pm0.01^{a,b}$	$5.51\pm0.42^{b,c}$	AF3	$0.15\pm0.01^{b,d,g,h}$	29.52 ± 0.24^{c}	PP3	0.15 ± 0.00^{c}	$5.59\pm0.65b^{b,c}$
90	PCAF4	$0.66\pm0.01^{g,h}$	32.53 ± 0.76^{d}	PCPP4	0.50 ± 0.00^{a}	$5.92\pm0.25^{c,d}$	AF4	$0.17\pm0.01^{h,i}$	$30.95\pm1.23^{c,d}$	PP4	0.17 ± 0.00^{g}	$5.62\pm0.79^{c,e}$
120	PCAF5	0.63 ± 0.01^{c}	37.07 ± 0.66^{e}	PCPP5	$0.56\pm0.00^{e,f}$	7.39 ± 0.23^{e}	AF5	$0.16\pm0.00^{c,d,e,i}$	32.34 ± 0.11^{d}	PP5	0.16 ± 0.00^{e}	5.66 ± 0.50^{e}
150	PCAF6	$0.64\pm0.01^{c,d,f}$	40.14 ± 1.54^{f}	PCPP6	$0.57\pm0.00^{d,f}$	14.09 ± 1.19^{f}	AF6	$0.19\pm0.01^{k,l}$	49.27 ± 0.81^{e}	PP6	0.15 ± 0.00^{d}	11.03 ± 0.62^{f}
180	PCAF7	0.60 ± 0.01^{b}	42.61 ± 0.54^{g}	PCPP7	0.55 ± 0.00^{c}	$21.59\pm1.86^{g,h}$	AF7	$0.20\pm0.01^{j,k}$	55.65 ± 0.55^{g}	PP7	0.15 ± 0.00^{c}	12.52 ± 1.29^{g}
210	PCAF8	0.71 ± 0.01^{i}	45.33 ± 0.53^{h}	PCPP8	0.55 ± 0.00^{c}	22.53 ± 0.90^{g}	AF8	$0.19\pm0.01^{j,l}$	$55.25\pm2.41^{g,f}$	PP8	0.16 ± 0.00^{f}	18.73 ± 0.88^{h}
250	PCAF9	$0.65\pm0.01^{d,e,h}$	48.88 ± 0.83^{i}	PCPP9	$0.56\pm0.00^{d,e}$	21.57 ± 0.69^{h}	AF9	0.14 ± 0.00^{a}	55.02 ± 0.71^{f}	PP9	0.14 ± 0.00^{a}	24.01 ± 0.70^{i}

(SAE) Sinapic acid equivalents. Mean ± SE; $n = 3$. In each column different letters mean significant differences ($p < 0.05$). The first enzyme of each combination was added at time 0 while the second one 120 min later.

substrates during gastrointestinal digestion (PP) and under an exogenous enzyme system (AF).

3.3. Antioxidant capacity profiles (ACP) for rapeseed protein hydrolysates

The possible influence of naturally-present phenolic compounds on the antioxidant behaviour of RPHs was studied through *in vitro* scanning of antioxidant capacity among RPHs prepared from RPC and RPI and the antioxidant activity observed in Caco-2 cells.

Important differences in overall antioxidant capacity between hydrolysates arose from performance and interactions (with one another or with other components present in the hydrolysates such as phenolics) of the constituting peptide. A mixture of peptides may exhibit certain antioxidant profile because it contains a high amount of fragments that works with a synergic or antagonic effect or contains few peptides with very high activity (Yu, Jawa, Pan, & Kastin, 2004). Therefore, in order to distinguish among representative antioxidant activity profiles of active peptides within RPHs, antioxidant capacity values were considered as observable characteristics of antioxidant behaviour and then were used as input to perform latent profile analysis (LPA). The aim of carrying out LPA was to identify and gather samples with similar performance of independent response variables (antioxidant capacity) in mutually exclusive groups, each one representing a type of the non-observable variable (the antioxidant capacity profile). A five-group model was preferred (model selection parameters presented in Table 3); it was assumed that the latent variable (unobservable) has 5 categories or types. Members of each group display a homogeneous behaviour for some of the methods used to evaluate antioxidant capacity (they match a rule that make a particular sample belonging to certain group); nonetheless, samples of different groups may share similar values of other independent variables. The combination of these common antioxidant capacity values that matched the rule is the distinctive profile or pattern depicted in Fig. 2.

From the 5 profiles, hydrolysates prepared by using PP were grouped into 3 ACP; limited hydrolysates (DH < 11) prepared from RPC (profile 1) exhibited distinctive average antioxidant capacity values: DPPH scavenging activity (23.34±0.40%), inhibition of Cu^{2+} catalized β-carotene oxidation (74.46±0.46%), β-carotene bleaching inhibition (84.39±1.19%), $ABTS^{\cdot+}$ scavenging activity (8.60±0.06 mM TEAC/g) and reducing power (0.65±0.01 Abs_{700nm}). Their PC-associated counterparts prepared from RPC were gathered into another group of profile 2 (Fig. 2b) that only shared inhibition of Cu^{2+}-catalysed β-carotene oxidation (61.29±0.78%), β-carotene bleaching inhibition (90.01±0.29%) and $ABTS^{\cdot+}$ scavenging activity (8.38±0.13 mM TEAC/g), values similar to those reported by Mäkinen et al. (2012).

Extensive hydrolysates prepared by using pepsin and pancreatin from both protein substrates belonged to the group of profile 4 (Fig. 2d) since they shared average values of inhibition of Cu^{2+}-catalysed β-carotene oxidation (72.97±0.88%), β-carotene bleaching inhibition (89.26±0.39%), Cu^{2+}-chelating activity (49.60 ± 0.88%) and $ABTS^{\cdot+}$ scavenging activity (9.27±0.03 mM TEAC/g). Thus, phenolic compounds had no influence on these samples' inhibitory activity of lipid oxidation.

On the other hand hydrolysates prepared with AF were gathered into 2 groups, the hydrolysates prepared from RPI shared profile 3 (Fig. 2c) with similar behaviour of DPPH scavenging activity (33.56±0.67%), cellular antioxidant activity (25.54±0.62%), Cu^{2+}-catalysed β-carotene oxidation (77.41±0.20%), β-carotene bleaching inhibition (79.02±0.75%) and Cu^{2+}-chelating activity (78.17±0.60%); all of them were also similar to distinctive profile 5 (Fig. 2e) of their phenolic-associated analogous characterised by cellular antioxidant activity (62.41±1.48%), Cu^{2+}-catalized

Table 2
Average molecular weight distributions and free amino acid content of rapeseed protein hydrolysates obtained from rapeseed protein concentrate (RPC) and rapeseed protein isolate (RPI).

Hydrolysis time	Rapeseed protein concentrate (RPC)								Rapeseed protein isolate (RPI)							
	60 min		120 min		150 min		240 min		60 min		120 min		150 min		240 min	
Enzymes	AMW distribution								AMW distribution							
	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%
Alcalase–flavourzyme	11.8–0.19 < 0.19	84.8 11.53	11-8-0.19 < 0.19	83.9 12.83	9.6–0.190 < 0.19	82.1 17.22	7.86–0.190 < 0.19	73.3 24.3	50–32.7 32.7–10.6 10.6–0.176 < 0.176	4 8.4 79.5 7.6	50–8.4 8.40.176 < 0.176	9 77.6 11.78	50–33.69 33.69–8.23 8.23–0.176 < 0.176	2 7.36 73.12 16.42	50–9.9 9.9–0.176 < 0.176	11.15 79.23 8.3
	Free amino acid content (% of total)								Free amino acid content (% of total)							
	HAA	0.20		0.65		1.72		3.20	HAA	1.41		1.26		3.14		3.81
	PAA	0.01		0.24		0.40		0.93	PAA	0.06		0.06		0.06		0.06
	(+)CAA	0.03		0.15		0.40		0.87	(+)CAA	0.24		0.44		1.35		2.26
	(−)CAA	0.03		0.17		0.19		0.57	(−)CAA	0.24		0.32		0.98		1.33
	AMW distribution								AMW distribution							
	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%	(kDa)	%
Pepsin–pancreatin	47.8–0.258 < 0.258	93 6.8	47.8–0.282 < 0.282	92.8 5.54	47.8–0.23 < 0.23	85.6 13.37	47.8–8.32 3.32–0.21 < 0.21	18.7 59.4 20.6	45–0.628 < 0.628	99.64 0	45–2.8 2.70–0.264 < 0.264	74.1 21.36 0.27	45–8.95 8.95–2.7 2.0–0.209 < 0.209	32.15 26.31 26.18 10.02	45–8.46 8.46–2.3 2.2–0.209 < 0.209	31.12 23.68 31.25 10.99
	Free amino acid content (per cent of total)								Free amino acid content (per cent of total)							
	HAA	0.21		0.49		2.89		4.50	HAA	0.12		0.17		3.86		4.31
	PAA	0.22		0.31		0.35		1.74	PAA	0.08		0.10		0.19		1.97
	(+)CAA	0.00		0.14		0.94		1.77	(+)CAA	0.01		0.03		0.45		0.60
	(−)CAA	0.09		0.23		0.30		1.07	(−)CAA	0.06		0.07		0.12		1.93

(HAA) Hydrophobic amino acids combine; Ala, Val, Ile, Leu, Phe, Trp, Pro and Met. (PAA) Polar uncharged amino acids combine Asx, Glx, Ser, Thr Tyr and Cys; ((+)CAA) Positively charged amino acids combine Hys, Arg, and Lys and ((−)CAA) Negatively charged amino acids combine Asx and Glx. The first enzyme of each combination was added at time 0 while the second one 120 min later.

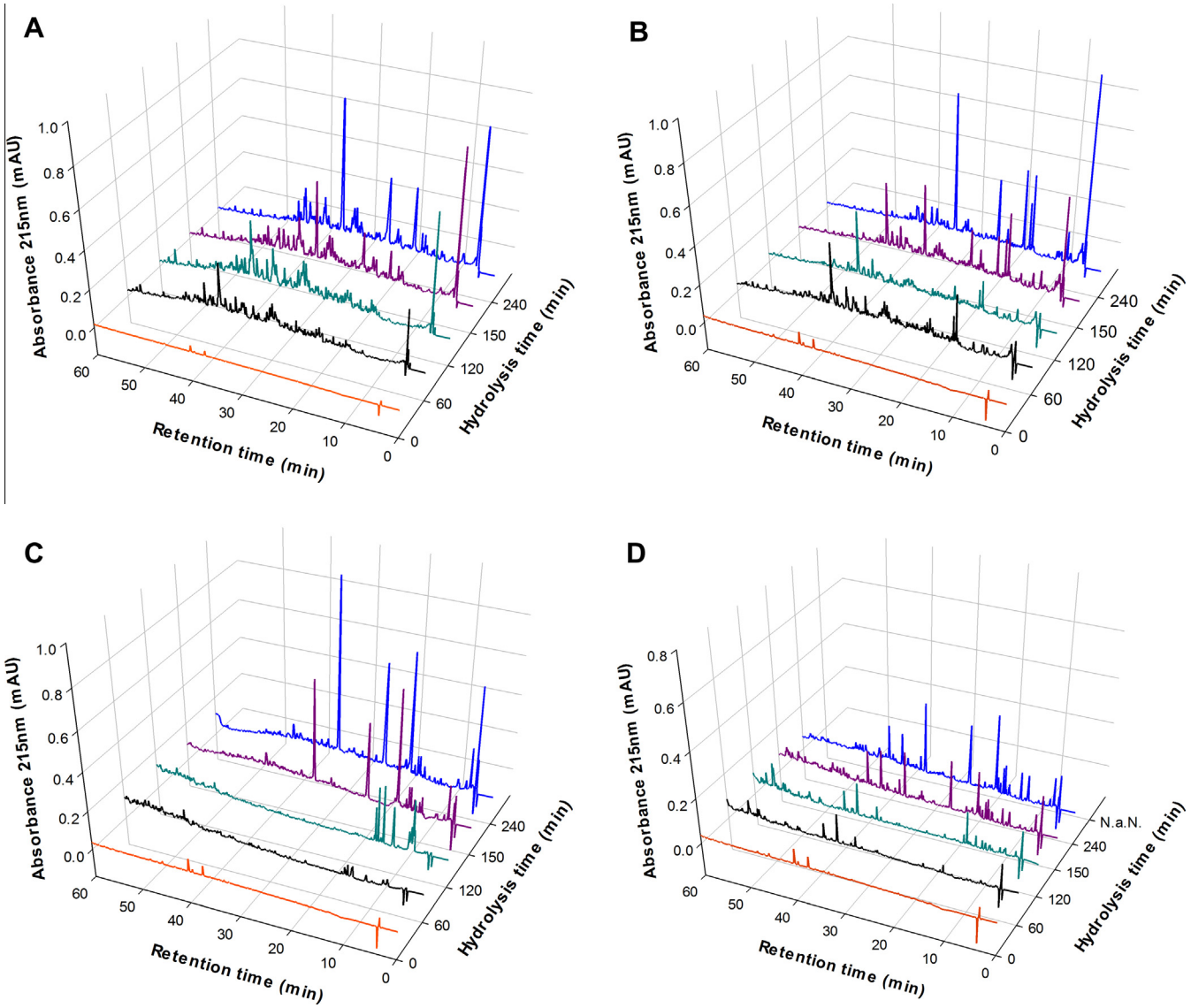

Fig. 1. Elution profile of hydrolysates separated by RP-HPLC. (A) Hydrolysates obtained with alcalase–flavourzyme from rapeseed protein concentrate (RPC), (B) hydrolysates obtained with alcalase–flavourzyme from rapeseed protein isolate (RPI), (C) hydrolysates obtained with pepsin–pancreatin from RPC, (D) hydrolysates obtained with pepsin–pancreatin from RPI. (The first enzyme of each combination was added at time 0 while the second one 120 min later.).

β-carotene oxidation (62.41±0.43%), β-carotene bleaching inhibition (91.75±0.22%) and Cu^{2+}-chelating activity (74.53±0.58%) independently of DH of mixtures inside these groups. Consequently, these ACP may be related to the specificity of the enzymes used in this study.

LPA allowed for identifying similar antioxidant behaviours among hydrolysates with different compositional characteristics; among them, the influence of minor antioxidant components (phenolics included) that interact with the peptides on ACP was different depending on the mixture characteristics. While in mixtures of peptides prepared with AF the DPPH scavenging activity and cellular antioxidant activity were the properties most affected by the presence of phenolics, for RPHs prepared with PP the influence was found only for limited hydrolysates. In contrast, PP mixtures of peptides with a DH higher than 11% shared the same ACP (profile 4) despite the presence of phenolics. Hence, in order to investigate on PC contribution to ACP of RPHs the rapeseed polyphenols extract (RPE) and sinapic acid (SA) as standard were assayed and compared with selected hydrolysates reduced in PC (based on the higher cellular antioxidant protection scores). EC_{50} values for antioxidant capacity are presented in Table 4. Significantly minor EC_{50} values for antioxidant capacity were found for PC (RPE and SA) in comparison to peptides, the values reported in these work are in agreement with previous reports for RPHs and RPE (Vuorela et al., 2005).

These results suggest that interaction of peptides with phenolic compounds diminish *in vitro* antioxidant capacity of RPHs, probably because such interaction occurs between residues that no longer are able to contribute to the antioxidant capacity and because the presence of phenolic compounds also affects protein solubility. Prigent et al. (2003) reported that the reaction of phenolic compounds with proteins may induce cross-linking of the proteins. These interactions also change the net charge in the protein molecules, which in turn affects the solubility of the derivatives. The secondary and tertiary structures of the proteins change as a result of these interactions, influencing the surface properties of the molecules by making them hydrophobic in nature.

On the contrary to some of *in vitro* determined antioxidant capacity values, higher cellular antioxidant protection was achieved with RPHs produced from RPC, suggesting possible

Table 3
Latent profile analysis and multinomial logistic regression analysis.

LPA model diagnostic and selection						
Parameters	Number of latent profiles (n)					
	n = 4		n = 5		n = 6	
p-Value	0.0025		0.0085		0.0127	
AIC	10020.032		9640.002		9557.045	
BIC	10165.169		9715.517		9763.937	
Log-likelihood	−4967.016		−4767.001		−4718.022	
Entropy	0.993		0.997		0.999	
Antioxidant capacity profiles characterisation						
Profile number	1		2	3	4	5
Observed frequency	30		36	54	42	54
Percent of total (%)	13.90		16.70	25.00	19.40	25.00
Samples	PP1, PP2,PP3, PP4, PP5		PCPP1, PCPP2,PCPP3, PCPP4, PCPP5, PCPP6	AF1, AF2,AF3, AF4, AF5, AF6, AF7, AF8, AF9	PP6, PP7, PP8, PP9,PFPP7,PFPP8, PFPP9	PFAF1, PFAF2, PFAF3, PFAF4, PFAF5, PFAF6 PFAF7, PFAF8, PFAF9
Mean physical and chemical characteristics						
TPC (gSAE/100 g)	$0.01\pm0.00^{b,c}$		0.53 ± 0.08^{e}	0.02 ± 0.00^{c}	0.24 ± 0.11^{d}	0.01 ± 0.02^{a}
DH (%)	4.90 ± 1.03^{a}		27.12 ± 5.94^{c}	36.51 ± 7.42^{e}	18.72 ± 1.86^{b}	33.29 ± 5.71^{d}
(+)CAA(g/100 g)	52.64 ± 0.07^{e}		48.68 ± 0.3^{b}	48.31 ± 0.97^{b}	48.66 ± 1.28^{b}	47.33 ± 0.84^{a}
HAA (g/100 g)	32.45 ± 0.14^{a}		34.99 ± 0.26^{b}	36.89 ± 0.69^{d}	35.84 ± 0.95^{c}	36.44 ± 0.88^{d}
PAA (g/100 g)	14.91 ± 0.10^{a}		16.33 ± 0.10^{b}	14.79 ± 0.33^{a}	17.60 ± 0.29^{c}	16.24 ± 0.22^{b}
Multinomial logistic regression						
Variable	Baseline: profile 2 (lower cellular antioxidant activity)					
	Profile 1		Profile 3		Profile 4		Profile 5	
	Log-odds ratio	p-Value	Log-odds ratio	p-Value	Log-odds ratio	p-Value	Log-odds ratio	p-Value
DH	0.437	0.999	0.77*	0	1	0.996	1.02	0.348
(+)CAA	4.794	0.998	266.94*	0	2.62*	0.035	50.58*	0
HAA	2.6E-10	0.996	0.33*	0	0.88	0.477	0.51*	0.001

For hypothesis test $H_0 = n - 1$, $H_a = n$. (AIC) Akaike information criterion. (BIC) Bayesian information criterion. (SAE) Sinapic acid equivalents. (HAA) Hydrophobic amino acids combine; Ala, Val, Ile, Leu, Phe, Trp, Pro and Met. (PAA) Polar uncharged amino acids combine Asx, Glx, Ser, Thr Tyr and Cys; ((+)CAA) positively charged amino acids combine Hys, Arg, and Lys. In each row different letters mean significant differences ($p < 0.05$). For multinomial logistic regression profile 2 (lower cellular antioxidant activity) was the baseline for odds calculations.

* Significant values ($p < 0.05$).

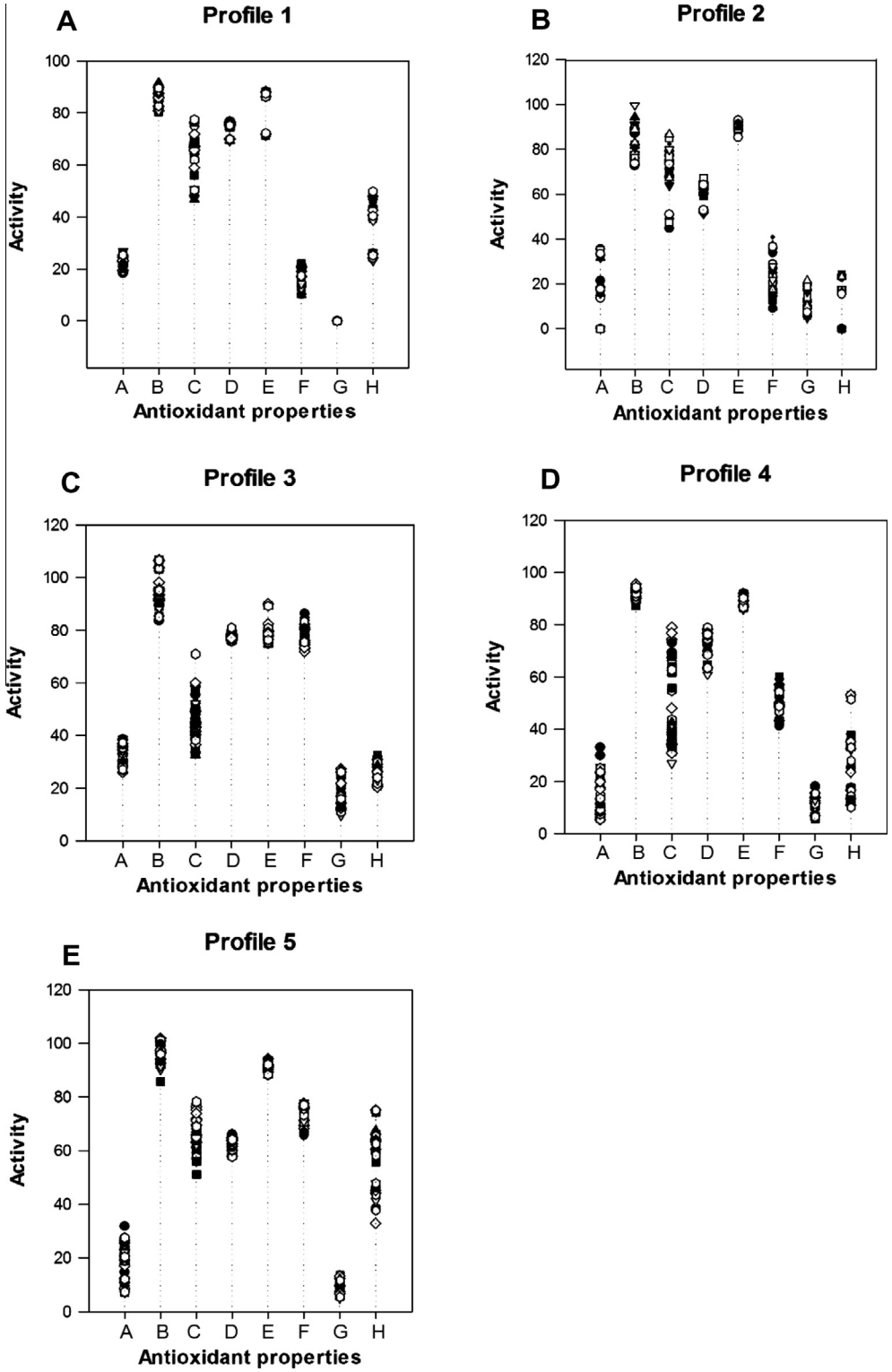

Fig. 2. Antioxidant capacity profiles (ACP). (A) DPPH radical scavenging activity (%), (B) $ABTS^{\cdot+}$ scavenging TEAC (mmol TE/g) $\times 10^{01}$, (C) Reducing power (Abs 700 nm) $\times 10^{02}$, (D) Cu^{2+} catalysed β-carotene bleaching method (%), (E) β-carotene bleaching method (%), (F) Cu^{2+} chelating activity (%), (G) Fe^{2+} chelating activity (%), (H) Cellular antioxidant activity (% AA).

synergistic effect of peptides and phenolic compounds. In addition transformations to peptides and PC carried out by cells might have produced metabolites with increased biological activity (Niki, 2010; van Duynhoven, Vaughan, Jacobs, Kemperman, & van Velzen, 2010). When assayed individually, RPE, SA or peptides, significant dose-dependent inhibition of ROS production is achieved for all samples; however, concentrations of peptides must be about 10-fold above to exert a similar effect than that exerted by SA or RPE. These results suggest that possible transformations and liberation of SA associated to peptides during extra or

Table 4
EC_{50} values for antioxidant capacity of rapeseed protein hydrolysates, rapeseed phenolic extract and sinapic acid.

Sample	ACP number	Radical scavenging activity		Reducing power EC_{50} (mg/mL)	Inhibition of β-carotene bleaching			
		DPPH EC_{50} (mg/mL)	$ABTS^{\cdot+}$ EC_{50} (μg/mL)		Catalysed by linoleic acid peroxidation products EC_{50} (mg/mL)	Catalysed by Cu^{2+} EC_{50} (mg/mL)	Cu^{2+} chelating activity EC_{50} (mg/mL)	Cellular antioxidant activity on Caco-2 cell culture EC_{50} (mg/mL)
AF3	3	2.18	0.08	4.53	0.03	0.05	0.05	6.68
AF5	3	2.31	0.08	5.00	0.02	0.09	0.03	6.84
PP4	1	2.20	0.09	1.61	0.47	0.02	1.05	4.90
PP8	4	3.77	0.08	2.18	0.02	0.04	0.41	7.89
RPE	–	0.002	0.002	0.12	0.001	0.01	0.20	0.87
SA	–	0.02	0.0005	0.10	0.005	0.73	0.05	0.50

(AF3) Rapeseed protein hydrolysate obtained by hydrolysis with alcalase during 60 min. (AF5) Rapeseed protein hydrolysate obtained by hydrolysis with alcalase during 120 min. (PP4) Rapeseed protein hydrolysate obtained by hydrolysis with pepsin during 90 min. (PP8) Rapeseed protein hydrolysate obtained by hydrolysis with pepsin during 120 min followed by 90 min of hydrolysis with pancreatin. (RPE) Rapeseed phenolics extract. (SA) Sinapic acid.

intracellular hydrolysis may be responsible for increased antioxidant protection found for RPHs prepared from RPC.

These results about the differences in antioxidant capacity values of different rapeseed hydrolysates are important since it has been postulated that oxidative stress is a very complex process that does not only involve the damage to macromolecules caused by free radicals but also the perturbation to the whole cellular redox state by different mechanisms (Niki, 2010). As explained by López-Alarcón and Denicola (2013), chemical and cell-based assays are influenced by different factors and the antioxidant capacity of a sample determined by cell-based assays does not correlate well to performance of *in vitro* assays. For rapeseed hydrolysates, the final antioxidant response at the cellular level essentially depended on the bioavailability of the bioactive peptides and minor compounds present and their synergistic interactions.

3.4. Multinomial logistic regression analysis

Members in each ACP pattern, may have heterogeneous composition characteristics as occur with the TPC in group 4 (Table 3) composed of hydrolysates prepared from both hydrolysis substrates; therefore, in order to prove theoretical hypothesis that relate some physical and chemical characteristics of RPHs with antioxidant behaviour TPC, DH, and AA content of RPHs were analysed through multinomial logistic regression by using the groups (patterns) obtained from LPA as a dependent variable (with 5 possible outcomes). Profile 2 (non-cellular antioxidant activity) was used as a basis for comparisons.

Significant contribution to the possibilities of showing profile number 3 in relation to show the base profile (profile 2) was found to be related to the DH, content of positively-charged residues and hydrophobic amino acids of members in this group. Possibilities to show pattern 3 (*vs.* pattern 2) increased in 23% with each additional unit in the DH. Whereas the possibilities of showing pattern 3 instead of pattern 2 for a sample with one additional unit of positively-charged residues are 266 fold higher than the possibilities of a sample with one unit less.

Positively charged amino acids were also significant for increasing the possibilities of displaying pattern 4; the possibilities for a sample to show this pattern instead of pattern 2 increased 2.62-fold with every additional unit of such amino acids.

Possibilities to show pattern 5 or pattern 3 (*vs.* pattern 2) are negatively influenced by hydrophobic amino acids; this possibilities decreased 50% with each additional unit of hydrophobic amino acids. The influence of the content of positively-charged amino acids may be affected by their frequency and position among peptide sequences into mixtures.

It was found that possibilities for a sample to exhibit profiles of antioxidant capacity with higher antioxidant protection in Caco-2 cells increased with each additional unit of positively-charged amino acids and diminish with each additional unit of hydrophobic amino acids. These results would provide meaningful information for further purification of antioxidant peptides based on a cationic enrichment criteria for mechanistic studies.

4. Conclusions

Distinctive antioxidant capacity profiles which describe specific antioxidant performance were established for different RPHs, the interaction of peptides with naturally-present phenolic compounds in RPC diminished *in vitro* antioxidant capacity of RPHs in comparison to phenolics-reduced hydrolysates or phenolic extract. However, antioxidant protection to Caco-2 cells increased in RPHs obtained from the substrate containing phenolics (RPC), as a consequence of a possible synergistic effect. The content of positively-charged amino acids and DH were compositional characteristics directly related to antioxidant capacity profiles with higher protection to Caco-2 cells.

Further research is needed for a suitable value-adding use of the rapeseed by-product through exploitation of its proteins for producing antioxidant hydrolysates. Rapeseed protein concentrate may be an adequate substrate for obtaining antioxidant hydrolysates aimed to contribute to physiological antioxidant systems; however, if antioxidant capacity is desired for a food matrix, hydrolysates obtained either from protein isolate or a phenolics extract would be preferred.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.12.063.

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