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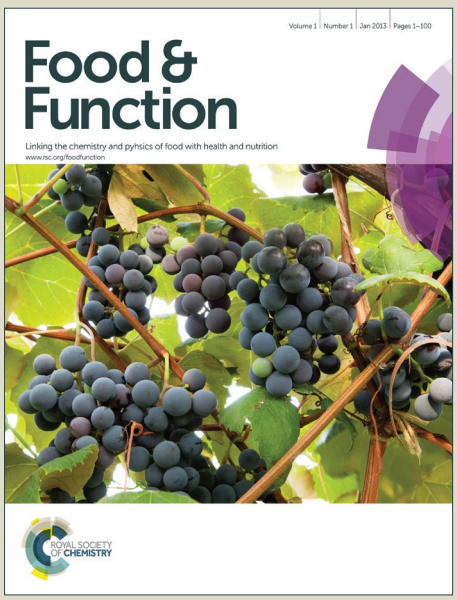

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PROTEIN ENRICHED PASTA: STRUCTURE AND DIGESTIBILITY OF ITS PROTEIN NETWORK

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Abstract

Wheat (W) pasta was enriched in 6% gluten (G), 35% faba (F) or 5% egg (E) to increase its protein content (13% to 17%). The impact of the enrichment on the multiscale structure of the pasta and on *in-vitro* protein digestibility was studied. Increasing the protein content (W- vs. G-pasta) strengthened pasta structure at molecular and macroscopic scales but reduced its protein digestibility by 3% by forming a higher covalently linked protein network. Greater changes in the macroscopic and molecular structure of the pasta were obtained by varying the nature of protein used for enrichment. Proteins in G- and E-pasta were highly covalently linked (28-32%) resulting in a strong pasta structure. Conversely, F-protein (98% SDS-soluble) altered the pasta structure by diluting gluten and formed a weak protein network (18% covalent link). As a result, protein digestibility in F-pasta was significantly higher (46%) than in E- (44%) and G-pasta (39%). The effect of low (55 °C, LT) vs. very high temperature (90 °C, VHT) drying on the protein network structure and digestibility was shown to cause greater molecular changes than pasta formulation. Whatever the pasta, a general strengthening of its structure, a 33% to 47% increase in covalently linked proteins and a higher β-sheet structure were observed. However, these structural differences were evened out after the pasta was cooked, resulting in identical protein digestibility in LT and VHT pasta. Even after VHT drying, F-pasta had the best amino acid profile with the highest protein digestibility, proof of its nutritional interest.

Introduction

Wheat pasta is a widely consumed staple food worldwide. As well as being a source of carbohydrates (74-77%, db) and proteins (11-15%, db), it has other interesting nutritional properties, notably its low glycemic index.[1] The structure of cooked pasta is usually described as a protein network with entrapped swollen starch granules. This specific structure, which is the result of successive changes that occur at different scales throughout pasta processing, has been shown to affect the digestibility of its nutrients, and their metabolic and health effects.[1,2]

Plants with high protein content such as legumes, or gluten and animal-based ingredients, such as egg white, are incorporated into pasta to enhance its nutritional or textural and cooking properties.[3-5] Enrichment with 10-20% wheat gluten has been shown to reduce cooking loss, increase the firmness of pasta, and to improve the encapsulation of starch, thereby reducing its accessibility to α-amylase.[6] Enrichment with 3-18% egg white has been shown to increase the firmness and elasticity[7,8] and to reduce the compressibility of cooked pasta.[9] Split pea,[10] faba bean,[10,11] fermented pigeon pea,[12] lupin,[13,14] black gram,[15] lentil, pea and chickpea[16] have all been used in different amounts (up to 50%) to enrich pasta to improve its protein content, its essential amino acid (EAA) score[11], especially lysine (lys) and to a lesser extent threonine (thr), its protein digestibility,[12] and to reduce glycaemia.[17] Legumes are also rich in dietary fibers, vitamins, minerals and carbohydrates.[4,18] However, high (>10-30%) legume substitution in pasta may weaken its protein network by decreasing covalent linkages, thereby impairing the overall quality of pasta.[14,18] The use of high temperature drying (>70°C) has been shown to improve pasta properties through the formation of a strong covalently linked protein network in wheat[19] and legume fortified pasta.[20] Severe thermal treatment has been shown to reduce the *in-vitro* digestion of protein and starch in wheat pasta.[21,22] In pasta fortified with 35% legume, the slowdown of *in-vitro* starch digestion due to high temperature drying was even more pronounced,[20] and was demonstrated *in-vivo* to improved digestive comfort and satiety after eating.[23] However, the

digestibility of the pasta protein network according to its protein composition or processing conditions has been less widely studied than starch digestibility. Few data[21,22,24] are available on the relationship between the structure and digestibility of the protein, especially in a multi-protein system pasta. No data are available on the simultaneous effect of protein content and composition and of the drying temperature on the digestibility of the protein in pasta. The aim of the present study on plant and animal protein enriched-pasta was thus to obtain further insight into the multiscale structural modifications in the protein network according to the level and nature of proteins and/or the drying diagrams used for pasta production. The effects of such structural changes on the *in-vitro* protein digestibility of pasta are discussed.

1. Material and methods

1.1. Material

Wheat (*Triticum durum*) (W) semolina, Faba bean (V*icia faba*) (F) flour, wheat (*Triticum aestivum*) gluten (G) powder and egg white (E) powder were provided by Panzani (Marseille, France), GEMEF industries (Aix-En-Provence, France), Syral, (Aalst, Belgium) and IGRECA (Seiche sur le Loir, France), respectively. The protein contents of all raw materials were analyzed using Kjeldahl procedure (NF V 03-050, 1970) with a conversion factor of 5.7 for W-semolina and G-powder and 6.25 for F-flour and E-powder. Their total starch content was determined using an enzymatic assay kit (Megazyme, Co. Wicklow, Ireland; AACC method 76-13.01). All analyses were conducted in duplicate.

1.2. Pasta manufacturing

All pasta was processed to produce spaghetti using a continuous pilot-scale pasta extruder (Bassano, Lyon, France). W-semolina was enriched with 6% G-powder, 35% F-flour or 5% E-powder to obtain 17% protein content in the pasta. This level is the highest that can be reached in F-pasta without introducing major difficulties in the pilot scale manufacturing process.[18]

W- and enriched-pasta were hydrated to 47% and 45%, respectively, and processed as described in Petitot *et al.,*[18,22] then dried at low temperature (LT-55 °C for 15 h) or at very high temperature (VHT-90 °C for 3 h) in a pilot-scale drier (AFREM, Lyon, France) to reach 12% moisture content. The diameter of the dry pasta was 1.56±0.03 mm. Two batches of each kind of pasta were produced and mixed into a single batch prior to pasta analysis. Pasta protein content of the final batch was measured using the Kjeldahl procedure with a conversion factor of 5.7 for W-semolina and G-powder and 6.25 for F-flour and E-powder according to their respective proportions in pasta.

1.3. Cooking quality of pasta

The optimum cooking time (OCT) of each kind of pasta was determined according to the approved AACC method 66-50.25. All analyses of cooked pasta were made on pasta cooked at OCT+1 min according to Petitot *et al.* [22] Cooking loss and water uptake of cooked (OCT+1 min) pasta were determined in triplicate, as previously described in Petitot *et al.*[18]

1.4. Rheological properties of cooked pasta

The textural properties of cooked (OCT+1 min) pasta were determined using a TA-XTplus (Stable Micro Systems, Scarsdale, USA) texture profile analyzer equipped with a Windows version of Texture Expert software package (Stable Micro Systems, Scarsdale, USA). Prior to measurement, the cooked pasta was equilibrated at ambient temperature for 10 min in a saturated vapor atmosphere container. Nine replicates of three different types of cooking were performed for each kind of pasta.

Pasta elongation. The TA-XTplus analyzer was equipped with tensile grips (ref. A/SPR, Stable Micro Sytems). The initial distance between the two tensile grips was 15 mm. The test was performed at a constant rate of deformation at 3 mm/s. Elongation (the ability of pasta to be elongated) was determined as the increase in pasta length (cm) until breakage, and was calculated according to the following equation:

$$\text{Elongation (\%)} = \frac{\text{Final lenth} - \text{Original lenth}}{\text{Original lenth}} \times 100$$

Pasta firmness. was determined based on the AACC approved Method 66-50,[25] expressed as the force (g) required to cut five strands of spaghetti positioned adjacent to another at a constant rate of deformation (0.17 mm/sec).

1.5. Pasta structure

Light microscopy of cooked pasta. Cooked (OCT+1 min) pasta sections (8 µm) were cut using a cryoprotector (Cellpath, Newtown, UK), and a microtome (Microm HM 520, Walldorf, Germany). The sections were stained with fast green and lugol as previously described in Petitot *et al.*[10] Bright field images were acquired using the multizoom AZ100M microscope (Nikon, Japan) equipped with a Nikon DSRiI (Nikon, Japan) color digital camera. Observations were made with a plan fluor 5x objective and a fixed optical zoom of 8 leading to a global magnification of 40x.

Protein size distribution of dry and cooked pasta. Sodium dodecyl sulfate (SDS) soluble proteins and dithioerythritol (DTE) soluble proteins (subjected to sonication) were extracted in triplicate from raw blends (used for pasta production) and from dried and freeze-dried cooked (OCT+1 min) pasta. All protein extracts were analyzed by size-exclusion high performance liquid chromatography (SE-HPLC) according to the modified method of Morel *et al.*[26] described in Petitot *et al.*[22] The protein fraction that was not extracted in either SDS or in DTE constituted the non-extractable fraction. Once corrected for the different solid-to-solvent ratios used during extraction, areas (in arbitrary units) of SDS-soluble and DTE-soluble proteins were summed and total extractable proteins are expressed as the percentage of the corresponding total area calculated for semolina (for W-pasta) or blends of semolina and high protein powders (35% F, 6% G or 5% E) for protein enriched-pasta.

Protein secondary structure of dry and cooked pasta. Infrared and fluorescence spectroscopies were performed on samples in the same physical state as those used for the study of *in-vitro* protein

digestibility. FTIR (Fourier transformed infrared) spectra were recorded on an FTIR Nicolet 6700 spectrometer, equipped with an attenuated total reflectance (ATR) Smart DuraSample IR accessory (ThermoScientific, U.K.) and a Mercury Cadmium-Telluride-High D detector. Interferograms (128) were collected at 2 cm^{-1} resolution and co-added before Fourier transformation. Spectra were recorded between 800 and 4000 cm^{-1}. In order to standardize their water content, all samples of both dry and cooked pasta were freeze-dried and pressed down against the diamond ATR surface. Nine spectra were recorded for each sample. Spectra were analyzed in the region of the amide I band (1,600-1,700 cm^{-1}) after baseline correction and normalization. Principal component analysis (PCA) was performed on both dry and cooked pasta spectra using the PLS-Toolbox v7.9 (Eigenvector Research, Inc) for Matlab (v10.0, Mathworks).

Protein tertiary structure of both dry and cooked pasta. Fluorescence spectroscopy was performed using a JASCO Spectrofluorometer FP-8300, equipped with spectra manager 2 (version 1.0). All dried and cooked freeze-dried pasta were excited at 290 nm and emission spectra were then recorded at room temperature (25±2 °C) from 300 to 390 nm with the scan rate of 10,000 nm/min at a constant 2.5 nm bandwidth for both excitation and emission, and a data interval of 0.5 nm. Maximum intensity spectra and their corresponding wavelengths (λ_{max}) were collected. Six replicates were performed for each sample.

1.6. Amino acid profile of raw material and dried pasta

The amino acide (AA) profiles of LT and VHT dry pasta and of the raw material used for their production were determined in duplicate at CIRAD (Montpellier, France) according to Moore *et al.*[27] using an AA analyzer (Biochrom 30+, Biochrom, France). The AA profile determined separately for each raw material (W-semolina, G- and E-powders and F-flour) was used to calculate the AA composition of blends used for pasta production. Essential AA scores (EAAS) correspond to the ratio of the amount of each AA in each sample to the amount of the same AA in

an ideal protein recommended for human adults (Anses, previously afssa).[28] EAAS have to reach 100% of the Anses recommendation for each AA to ensure optimal use of the protein.

1.7. *In-vitro* protein hydrolysis of cooked pasta

In-vitro protein digestion of pasta was performed in triplicate according to Pasini *et al.,*[29] using pepsin (700 U/mg protein, P7125, Sigma) and Pancreatin (Sigma catalog n° P7545). Both reactions were stopped after 30 min of pepsin hydrolysis or after 30 min pepsin+180 min of pancreatic digestion by adding one volume of 20% (w/v) trichloroacetic acid (TCA). The amount of free amino group was determined on digestion extracts (supernatant) based on the ninhydrin method.[30] The degree of hydrolysis (°H) was calculated as previously described by Petitot *et al.*[22]

1.8. Statistical analysis

All data (except for FTIR spectroscopy described in the spectroscopy method section) were subjected to analysis of variance (two-way ANOVA) using "formulation" and "drying" as factors. ANOVA was followed by the Fisher's least significant difference (LSD) test to compare means at the 5% significance level, using Statistica 8.0 software (Tulsa OK, USA).

2. Results

2.1. Composition of raw material and pasta

The composition of the raw material and their blends used for pasta production is provided in table 1. Starch was the main constituent of W-semolina and to a lesser extent of F-flour, while proteins were the main constituents of G- and E-powders. The enrichment of W-semolina with 6% G-, 5% E-powders or 35% F-flour increased the protein content of the blend by 30%. G-, F- and E-proteins contributed 28%, 50% and 27%, respectively, of the total protein content of the respective blends (table 1). Protein enrichment of W-semolina reduced the proportion of starch by 5% in the G- and E-blends and by 9% in the F-blend. The protein content of dry pasta determined by Kjeldahl's method was in agreement with the calculated contents, i.e. 13.15±0.03% (db) for wheat pasta and

16.96±0.15% (db) for all enriched-pasta whatever the drying profile (LT or VHT) used. Considering the contribution of each added protein to the total protein content in the blend, and the sulfur AA content of each raw material, the E- and to a lesser extent G-blend, were richer in sulfur AA and the F-blend poorer in comparison to W-semolina (table 1).

2.2. Effect of formulation and drying temperature on the cooking properties of pasta

The main effects of pasta formulation, drying and their interactions on pasta cooking properties are listed in table 2. Cooking loss and water uptake were significantly affected by pasta formulation. All protein-enriched pasta had lower water uptake as already reported in 15-35% legume (split pea, faba bean and pea)[11,18,31] and 9-20% gluten pasta.[32] This could be linked to the lower starch content in all enriched-pasta, and to the introduction of legume starch in F-pasta. In legume enriched-pasta (20 to 60% green gram), starch was previously shown to acquire a different amylograph profile from wheat pasta starch,[33] which could play a role in reducing water uptake in F-pasta. Conversely, no decrease in water uptake was reported in rice pasta enriched with 15% liquid egg albumen.[34] The enrichment of pasta with 6% gluten decreased its cooking loss by 11%. This is consistent with the results of Fardet *et al.*[6] (22% decrease after the addition of 10% gluten to wheat-pasta). No significant modification of this parameter was observed in LT E-pasta. Particle losses in cooking water only increased (by 13%) in F-pasta. Most authors reported 7-10% cooking losses in legume (faba bean, lupin, pea, lentil and chickpea) enriched-pasta[14,16,18] when the substitution level was ≥30%. Cooking losses were more affected by pasta drying temperature than by formulation (*F*-values in table 2). A slight but significant impact of interaction (formulation×drying) was also observed. Cooking loss decreased by 20% and 29% in VHT F- and VHT E-pasta, respectively, compared to LT dried pasta, without affecting W- and G-pasta. No modification in water uptake was observed with an increase in the drying temperature.

2.3. Effect of formulation and drying temperature on the rheological properties of pasta

Pasta firmness and elongation, which are good indicators of pasta structure at the macromolecular scale, are presented in table 2. The majority of textural properties of cooked pasta were significantly affected by pasta formulation ($p<0.05$). A marked increase in pasta firmness (by 38% and 74%, respectively) was observed in LT cooked G- and E-pasta in comparison to W-pasta. This was accompanied by a 16% increase in elongation in E-pasta. Similar improvements in pasta texture (firmness/compressibility and elasticity) have been reported in 3-18% egg albumen enriched wheat[8,9] and oat pasta,[8] and in 10-20% gluten enriched-pasta.[6] Enrichment of pasta with F drastically altered (by 32%) its elongation without affecting its firmness, when dried at LT. Zhao *et al*.[16] and Rayas-Duarte *et al*.[14] also reported no change in the firmness of 5-30% lupin, yellow pea and chickpea enriched-pasta. Reduced elasticity in 35% faba and 30% pea enriched-spaghetti was recorded by Petitot *et al*.[18] and Padalino *et al*.[31] Drying temperature had a significant effect on the rheological properties of the pasta, even if the effect was lower than that of the formulation (F-value in table 2). VHT generally increased pasta firmness and elongation with no interaction with formulation.

2.4. Effect of formulation and drying temperature on the structure of dry and cooked pasta

Microstructural scale, light microscopy of cooked (OCT+1 min) pasta. The microstructure of all LT-cooked pasta is presented in figure 1 as parts of a diagonal cross section observed by light microscopy. Starch granules colored bluish-purple are surrounded by the protein network stained green. At this scale, the increase in protein content in W- vs. enriched- (G, F and E) pasta was not clearly visible. In all pasta, a gradual increase in starch swelling from the core to the external region resulted in three main regions, as already reported by several authors.[10,35] The protein network appeared to be tighter in the core of G- and E- than in F-pasta. It was possible to differentiate faba bean starch (oval) from wheat starch (elongated) by their shape in the pasta core. In the intermediate region, the starch granules seemed larger and well swollen in all pasta except for E-pasta. In the external region of all pasta, as a result of the high exposure to water, the starch

granules were highly swollen or had even disintegrated, creating many empty protein cavities. However, no match could be established between the cooking losses measured on pasta (table 2) and their external state. Whatever the pasta formulation, no effect of VHT drying was observed on pasta microstructure (data not shown). This could be related to the same level of water uptake in LT vs. VHT pasta, whatever the formulation (table 2).

Molecular scale, Protein aggregation by SE-HPLC. Results of protein solubility in SDS and DTE (after sonication) in both dry and cooked pasta are presented in figure 2. The SDS-soluble fraction presented weakly linked (electrostatic, hydrophobic and hydrophilic) proteins; the DTE-soluble fraction contained disulfide bonded proteins. Proteins that were linked by other covalent interactions than disulfide bridges (i.e. isopeptide bonds) were considered to be the non-extractable fraction. In all LT dry pasta (figure 2A), more than 65% of proteins were soluble in SDS. The remaining proteins were linked by disulfide bonds (29, 32, 18 and 28% in W-, G-, F- and E-pasta, respectively). Enrichment of pasta with F-flour whose proteins were 98% SDS-soluble (results not shown) resulted in a noticeable (11%) increase in the weakly linked proteins counterbalanced by a decrease in the DTE-soluble proteins as previously reported by Petitot *et al.*[10] Conversely, the enrichment of pasta with G-powder, which contained 32% of large protein aggregates in SDS-soluble protein fraction vs. only 28% in semolina (result not shown), led to a slight increase in covalently linked proteins in G-pasta. The addition of E-proteins (98% SDS-soluble as F-proteins, result not shown) did not increase SDS-soluble proteins in E-pasta. This could be explained by the greater ability of E-protein (in comparison to F-proteins) to form DTE-soluble protein, which could counterbalance the SDS-solubility of E-pasta proteins. This could be due to the high sulfur AA content in E- vs. F-blends used for pasta production (table 1). The temperature (LT vs. VHT) used to dry pasta had a greater impact on protein linkage in pasta than formulation (*F-value*=17,959 and 71 for drying and formulation effects, respectively; data not shown). VHT drying (figure 2B) drastically decreased the SDS and increased DTE protein solubility in all pasta. The formation of

covalently aggregated proteins was already demonstrated in high temperature (>70°C) dried wheat[19,21,22] and legume pasta[20] and in pasteurized (95°C) fresh egg pasta.[36] The effect of drying temperature we observed in our results varied with the protein used for pasta enrichment (*F-value* of interaction between drying and formulation=39; data not shown): The evolution of protein behavior with an increase in drying temperature was more intense in E- than in the other pasta (60% decrease in SDS-soluble and 47% increase in DTE-soluble protein) due to its higher sulfur AA content (table1). Proteins in the other (W-, G- and F-) pasta responded in a similar way to the increase in drying temperature (41-47% decrease in SDS- and 33-39% increase in DTE-soluble proteins). We can therefore conclude that the effect of VHT treatment on the creation of DTE-soluble proteins in F-pasta was as efficient as in W- and G-pasta. However, even dried at VHT, the protein network of F-pasta remained the weakest, with the highest proportion of SDS-soluble proteins, the lowest proportion of DTE-soluble proteins and almost no non-extractable proteins, as already reported in the literature.[10] Results of protein aggregation in cooked pasta are presented in figures 2C and 2D. Cooking drastically reduced the difference in protein aggregation between LT and VHT dried pasta (*F-value of drying*=17,959 and 94 before and after cooking, respectively; data not shown), while those related to pasta formulation remained the same or even increased after cooking (*F-value*=71 and 100 before and after cooking, respectively; data not shown). Cooking led to a 3-fold increase in the percentage of disulfide bonds in all LT-pasta and to a 1.2 fold increase in all VHT-pasta. Non-extractable proteins were slightly more numerous in G- and E-cooked pasta. Cooking did not create additional non-extractable proteins when pasta was previously dried at VHT. Even if cooking increased soluble-DTE in F-pasta, the weakly linked proteins were always twice as numerous in this cooked pasta in comparison to W-, G- and E-pasta whatever the temperature used for drying.

Protein secondary structure by FTIR spectroscopy. Mid-infrared spectroscopy was used to evaluate the protein secondary structure of the pasta in the amid I spectral region,[37] even if some

contribution of the AA side chain has already been observed in this spectral region in wheat protein.[38] Considering the minor change in spectral intensities, a PCA was performed[39] using the spectra from both dry and cooked pasta. Figure 3A shows the projections of different dry pasta spectra on the first two axes (PC1 and PC2). Scores on PC1 (75.7% of the total variation) separated VHT pasta with positive values from LT pasta with negative values, whatever the formulation of the pasta. PC1 loading showed a positive peak at 1,626 cm^{-1} and a main negative peak at 1,656 cm^{-1} (figure 3B). VHT pasta spectra thus differ from LT spectra in the higher intensity of the absorbed band at 1,626 cm^{-1} in comparison to 1,656 cm^{-1}. These two peaks were linked to β-sheet and α-helix (with contribution of random structures), respectively.[38,40] Increasing the pasta drying temperature, thus, increased β-sheet at the expanse of α-helix and random coil structures of proteins. Our result are in agreement with those observed in heat treated (25-100 °C) ovalbumine,[41] legume (*Phaseolus vulgaris* globulins and isolate)[42,43] and 47% hydrated gluten.[40] The PC2 (16% of the total variation) axis separated the pasta spectra into two groups based on pasta formulation (figure 3A). The first group comprised W- and G-pasta (with negative values) and the second one F- and E-pasta (with positive values). PC2 loading (Figure 3B) showed a positive peak at 1,637 cm^{-1} attributed to β-sheet structures, and a negative peak at 1,608 cm^{-1} attributed to glutamine side chain vibrations[38]. Enrichment of pasta with E-powder or F-flour led to an increase in β-sheet structures in comparison to W- or G-pasta, in which the contribution of glutamine side chain vibrations was greater. Higher glutamine side chain vibration in W- and G-pasta was in accordance with the higher amount of this residue analyzed in the corresponding raw materials: 303 and 229 mg/g of W- and G-protein, respectively vs. 79 and 71 mg/g of F- and E-proteins, respectively (result not shown). Regarding the respective variance of PC1 (75.7%) and PC2 (16.0%), the effect of the drying temperature on the secondary structure of dry pasta observed by FTIR was in fact greater than the nature of the protein used to formulate the pasta, as already observed by SE-HPLC analysis. The spectra of the cooked pasta were analyzed in the same way as those of the dry pasta

(Figures 3C and 3D). Two groups of spectra can easily be identified according to PC1 (73.7%) and PC2 (20.6%): the W- and G-pasta spectra had lower PC1 and higher PC2 scores than F- and E-pasta spectra. Looking at PC1 and PC2 loadings (Figure 3D), spectral regions involved in the distinction between (W/G) and (F/E) pasta are observed at 1,610 and 1,628-1,636 cm^{-1} (related to glutamine side-chain and β-sheet vibration, respectively), as observed for dry pasta. The effect of the formulation is thus mainly related to the nature of protein used for enrichment and the relative amount of glutamine AA it contains. No clear difference between VHT and LT spectra was observed within each group (figure 3C), in contrast to Bock *et al.*[44], who distinguished pasta dried at a low temperature (60°C) from pasta dried at a high temperature (85°C) by a higher β-sheet and lower β-turn structures in cooked pasta.

Protein tertiary structure by front face fluorescence spectroscopy. Intrinsic protein fluorescence is related to the presence of aromatic AA, notably tryptophan (Trp), whose emission is highly sensitive to its local environment. Trp fluorescence has been used to monitor the change in protein tertiary structure in complex food system.[45,46] The maximum intensities and their corresponding wavelengths (λ_{max}) of both dry and cooked pasta are presented in table 3. The λ_{max} of all the pasta was around 330 nm, indicating that the tertiary structure of the proteins in the pasta created a more hydrophobic environment around Trp,[47] probably related to the hydrophobicity of Trp microenvironment in gluten protein.[48] Similar λ_{max} values were observed by Karoui *et al.*[46] in wheat pasta. To our knowledge no study has reported the fluorescence properties of Trp in legume or egg enriched-pasta. However, a λ_{max} values around 330 nm would be expected for F-pasta, as the λ_{max} of legumin (11S) and vicilin (7S), the main storage proteins in faba bean seed and wheat proteins, were 320-329 and 330 nm, respectively.[48,49] Neither VHT drying nor cooking changed the hydrophobicity of Trp microenvironment whatever the pasta considered. Trp emission intensities in both dry and cooked pasta were also analyzed (table 3). In dry pasta, a significant effect ($p<0.05$) of the drying temperature was observed. VHT drying led to a drastic decrease in emission

intensity whatever the pasta considered. Fluorescence quenching by disulfide bonds could be involved, as already reported in gluten after heating (at 70 °C) and cooling steps[48] and in commercial pasta dried at high vs. low temperatures.[45] The degree of decrease in emission intensity caused by drying temperature also differed according to the formulation of the pasta (significant effect of interaction). Emission intensity decreased 2.2 times in F- and E-pasta vs. only 1.6 times in W- and G-pasta. Even less pronounced than the effect of drying, pasta formulation had a significant impact on emission intensities (see F-values in table 3). The enrichment of LT dried pasta with F-flour resulted in a drastic (50%) decrease in emission intensity, whereas enrichment with E-powders led to a slight increase (7%) in fluorescence intensity. No significant change in this parameter was observed in G-pasta. These differences in emission intensities due to pasta formulation could be related to the difference in protein structure (notably the quantity of disulfide bridge which acts as a quencher of Trp residues), without neglecting the possible effect of the AA composition (notably the amount of fluorescent residues: 9, 12 and 16 mg/g for F-, W- and E-proteins, respectively).[50] After cooking, the effect of drying temperature, formulation and their interaction on emission intensity was still observed ($p<0.05$). As observed in dry pasta, the spectra of cooked VHT had a lower emission intensity than the spectra of LT ones.

2.5. Impact of formulation and thermal treatment on the nutritional quality of pasta

AA profile. Total EAA and their scores (EAAS), based on Anses recommendations,[28] in blends of raw materials used for pasta production are listed in table 4. Incorporation of G- or E-powder or F-flour in W-semolina resulted in an increase in total EAA of 3, 14 and 21% respectively, counterbalanced by a decrease in DAA compared to W-semolina. Low AA scores for lys and to a lesser extent for thr were observed in W-semolina (respectively 56 and 90%) and G-blend (respectively 51 and 95%), both these AA being deficient in wheat protein.[51] W-semolina enrichment with 5% of E-powder may make it possible to recover the required amount of thr and to increase the lys score to 81%. The adequate lys AA score was only achieved in the F-blend (lys

score: 107%). Pasta processing and drying at LT reduced lys scores in all the pasta to a similar extent (around 20% loss) compared to blends of raw materials but without decreasing their thr scores. EAAS still conformed with anses recommendations when the pasta was dried at VHT, except for lys in all pasta, thr in W- and G- pasta and Ile in F-pasta. Concerning lys content, increasing the pasta drying temperature from 55 to 90 °C, only affected F-pasta (14% decrease in lys in VHT vs. LT drying).

Protein digestibility of cooked (OCT+1 min) LT and VHT pasta. The amount of hydrolyzed protein after 30 min action of pepsin on LT and VHT cooked pasta is listed in table 5. The pasta proteins remained slightly digested (mean °H 4-6%) by pepsin, with no significant effect of the drying profile (p-value>0.05) as already reported by Petitot *et al.*[22] in wheat pasta. ANOVA revealed a significant effect of pasta formulation (p-value<0.05) with no significant effect of interaction with drying temperature. Pasta protein enrichment with E-powder and especially with F-flour led to a significant increase in the degree of hydrolysis by pepsin in comparison with that in W-pasta (mean °H value of 4.97, 6.40 and 3.82%, respectively) whatever the drying temperature used. No statistical differences in °H were recorded when the pasta was enriched with G-powder. After 180 min of additional pancreatic hydrolysis, pasta proteins were noticeably digested (mean °H of 39-46%), and digestion appeared to be significantly affected by the formulation, with an interaction between formulation and drying. G-pasta was less digested than W-pasta (mean °H of 39.24 and 42.36%, respectively). Protein hydrolysis was still higher in F- and E-pasta compared to W-pasta (mean °H of 46.22, 44.28% vs. 42.36%, respectively). Our results are consistent with *in-vivo*[12] and *in-vitro*[14] studies on 10% legume (fermented pigeon pea and lupin) enriched-pasta. To the best of our knowledge, there is no data in the literature on the impact of structural variation of E-pasta on its protein digestibility.

Even after the pancreatic phase, we reported no impact of drying temperature on protein digestibility. In agreement with our results, no influence of the drying profile of pasta on its

digestibility was reported in W-pasta dried at or below 90 °C.[21,22] Only drying at VHT (90 °C) applied as a post treatment (after low temperature drying)[22] or drying above 110-180 °C[21] reduced the digestibility of wheat pasta by 14% and 37% respectively.

3. Discussion

The primary objective of this study was to assess the impact of pasta protein enrichment on its structure and nutritional properties. Increasing the protein content of pasta from 13% (W-pasta) to 17% (G-pasta) reduced its water uptake and increased its firmness. This is in agreement with the results of Sissons *et al.*[32] who reported that a coupled decrease in gluten and increase in starch content increased pasta water absorption, making it softer. G-pasta underwent less cooking loss. The particular structure of its protein network, notably its higher covalently linked protein network, could partly explain this decrease in cooking loss, and may have reduced the degree of protein hydrolysis in G-pasta. The change in pasta structure and protein digestibility obtained by increasing protein content from 13% (W-pasta) to 17% (G-pasta) were less pronounced than changes occurred in pasta when gluten was replaced by egg or faba proteins. F-pasta enrichment gave the highest lys score (86% vs. 41% and 64% for G- and E-pasta, respectively), even with high temperature drying. Pasta texture has been shown to be highly dependent on the nature of the protein used for enrichment. E- and G-pasta were characterized by a higher firmness score with a better resistance to elongation (for E-pasta) and less cooking loss (for G-pasta) in agreement with previous studies on 10-20% gluten[6] and 3-15% egg[9,34] enriched-pasta. Conversely, F-pasta had a weakened texture and greater cooking loss, as previously demonstrated by Petitot *et al.*[18] G- and E-pasta presented a more compact microstructure in the center, and, in E-pasta, even in intermediate regions compared to the open microstructure of the core of F-pasta. All these differences in cooking loss and in the textural and microscopic properties of G- and E- vs. F-pasta could be linked both to the molecular properties of each protein used for the enrichment and to the contribution of each added protein to the total protein content in pasta. Indeed, proteins of F-flour

composed of albumins and globulins,[49] are 98% SDS-soluble with a low sulfur AA content (17 mg/g protein) and represented 50% of the total proteins in F-pasta (table 1). F-proteins diluted gluten network and reduced the opportunity for disulfide crosslinks to be formed, thereby weakening the protein structure in both dry and cooked F-pasta. Conversely, G- and E-proteins represented 28 and 27% (respectively) of the total protein content of enriched-pasta and both (but especially E-proteins) possessed a higher sulfur AA content (35 and 59 mg/g protein for G- and E-proteins, respectively) able to form disulfide bonds during drying and cooking, thereby strengthening the protein network. In addition to the composition of the proteins, which considerably affected pasta protein digestibility, and among the protein structure parameters we explored, the degree of protein hydrolysis in cooked protein enriched G-, E- or F-pasta appeared to be more related to the percentage of protein covalently linked than to its secondary and tertiary organization. Indeed, all pasta displayed an identical Trp environment hydrophobicity (λ_{max} at 330-335 nm). In addition, F-pasta proteins, which contained the highest β-sheets but the lowest covalently linked proteins, resulted in the highest degree of protein hydrolysis. Conversely, G- and E-pasta proteins were more covalently aggregated but G-pasta contained less β-sheet structure, while E-pasta contained the same amount as F-pasta proteins, and both were less hydrolyzed than F-pasta.

In the second step of this study, the effect of drying temperature on pasta structure was investigated on dry and cooked pasta as a function of the protein (G, E or F) used for their enrichment. Considering dry pasta, ANOVA analysis of the results of SE-HPLC, FTIR and fluorescence spectroscopy revealed that more molecular rearrangements of proteins were caused by the increase in drying temperature (55 vs. 90°C) than by the change in pasta formulation. The denaturation of protein by VHT drying led to extensive β-sheet formation probably at the expanse of α-helix unfolding. The resulting structure was stabilized by the formation covalent bridges leading to high covalent protein aggregation in all VHT dry pasta, as already reported in gluten proteins[40,52] and in

wheat pasta subjected to a severe hydrothermal treatment (60-100 °C).[22] The extensive formation of disulfide bonds was responsible for higher Trp emission quenching in VHT pasta in comparison to pasta dried at LT, in agreement with the results of Bonomi *et al.*[45] When G-, E- and F-pasta were cooked, the differences between LT and VHT pasta concerning their protein secondary structure and the density of the covalent protein linkages presented above were drastically reduced. Only a difference in the protein tertiary structure between VHT and LT cooked pasta remained (higher fluorescence quenching in all VHT vs. LT cooked pasta). As this fluorescence quenching was not associated with an increase in covalent bonds between proteins, it may be associated with a different molecular position of disulfide cross-links depending on the temperature used for drying the pasta before the cooking step. Disulfide bridges were probably closer to Trp residues in pasta dried at VHT, reflecting a more compact local Trp environment than in pasta dried at LT. These conformational changes in the protein network in pasta dried at VHT vs. pasta dried at LT were accompanied by an improvement in their firmness and elongation when cooked. Unlike the effect of formulation, these changes in the protein network structure observed at supramolecular scale were not related to a difference in the microstructure of VHT vs. LT pasta, and did not lead to any difference in protein digestibility. However, VHT drying, although beneficial for the rheological and cooking properties of pasta, decreased the lysine content of dry F-pasta by 14% compared to the same pasta dried at LT, which could negatively affect lysine release and bioavailability.[53] Interestingly, despite this loss, VHT F-pasta kept a higher lys score than G- and E-pasta. In addition, no alteration in the *in-vitro* protein hydrolyses was caused by VHT drying making F-pasta interesting from a nutritional point of view. It is now necessary to confirm whether this behavior is maintained *in-vivo*.

4. Conclusion

The present investigation highlighted the impact of plant (i.e. gluten and faba bean) or animal (egg) protein enrichment on pasta structure, with particular emphasis on its protein network, and on the

nutritional quality of the pasta. The protein network formed by the addition of egg was tight, β-sheet structured and stabilized through covalent bounds leading to improve textural and cooking properties of the pasta. Gluten or faba bean enrichments resulted in two distinct pasta structures. Like egg proteins, gluten enrichment improved the textural and cooking properties of the pasta by increasing protein covalent links without favoring β-sheet formation. Conversely, even if, like egg, faba bean enrichment of the pasta promoted β-sheet structure, it decreased covalent stabilizing bonds thereby altering pasta textural properties and cooking loss. High drying temperature of faba bean pasta could help recover textural properties, decrease cooking loss and bring them close to those of wheat pasta. In comparison to egg pasta, faba bean pasta presented a better amino acid profile, with a high lysine content even when dried at very high temperature, and higher protein digestibility proof of its nutritional interest.

5. Abbreviations

W, wheat; G, Gluten; F, Faba bean, E, Egg; LT, low temperature; VHT, very high temperature; OCT, optimal cooking time; AA, amino acid ; EAA, essential amino acid; EAAS, essential amino acid score; DAA, dispensable amino acid. His, histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Thr, Threonine; Trp, Tryptophan; Val, Valine; °H, degree of hydrolysis.

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Tables:

Table 1. Composition of wheat- (W) semolina, gluten- (G) powder, faba bean- (F) flour and egg- (E) powder, and the blends of 6%G+94%W (G-blend), 35%F+65%W (F-blend), and 5%E+95%W (E-blend) used for pasta production.

Raw material	**Composition**			
Pure	W-semolina	G-powder	F-flour	E-powder
Starch (%, db)	77.8 ± 0.6	10.6 ± 0.1	57.6 ± 0.3	na[a]
Protein (%, db)	13.1 ± 0.1	79.8 ± 0.1	24.0 ± 0.1	90.9 ± 0.4
Sulfur AA (mg/g protein)	30.2	34.9	17.4	58.7
In blend[b]		G-blend	F-blend	E-powder
Starch (%, db)		73.8	70.7	73.9
Protein (%, db)		17.1	16.9	17.0
Supplemented protein (% of total protein)[c]		28.0	49.7	26.8
Sulphur AA (mg/g protein)		31.5	23.8	37.8

[a]Not analyzed.

[b]Result obtained by calculation; G-blend: 6%G+94%W, F-blend: 35%F+65%W and E-powder: 5%E+95%W.

[c]Contribution (%) of supplemented protein (G, F or E) as a proportion of the total protein content in each blend.

Results are means of 2 replicates.

Table 2.Results of two way analysis of variance and of an LSD test of the cooking and rheological properties of wheat- (W) pasta and gluten (G), faba bean (F) and egg (E) enriched-pasta dried at low (LT) and very high temperature (VHT) and cooked to optimal cooking time +1 min.

Pasta properties	Mean effects[a]								*F-value*[b]		
	Pasta formulation	W		G		F		E				
	Drying temperature	LT	VHT	LT	VHT	LT	VHT	LT	VHT	Formulation	Drying	Interaction[c]
Cooking	Cooking loss (%, db)	7.5 b	6.9 ab	6.7 a	6.7 ab	8.5 d	6.8 ab	7.2 ab	5.1 c	11	30	7
	Water uptake (%, dry pasta)	186 c	181 c	174 b	173 b	165 a	165 a	163 a	164 a	48		
Rheological	Firmness (g)	493 a	558 b	680 c	852 d	499 a	552 b	857 d	992 e	594	180	
	Elongation (%)	339 a	353 ab	368 ab	452 c	230 d	324 a	394 b	446 c	26	23	

[a]Means in the same row with the same letter are not significantly different ($p>0.05$).

[b]*F-value* is given only when the effect was statistically significant.

[c]Interaction between drying and formulation.

Results are means of 3 replicates for cooking properties and of 9 replicates for rheological properties.

Table 3. Tryptophan maximum emission wavelengths (λ_{max}) and corresponding fluorescence intensity of wheat- (W) pasta and gluten (G), faba bean (F) and egg (E) enriched-pasta dried at low temperature (LT) and very high temperature (VHT), in the dry state or after cooking to optimal cooking time +1 min.

Pasta formulation and drying		λ_{max} (nm)[a]		Max. intensity (a.u.)[b]	
		dry	cooked	dry	cooked
W	LT	332	331	645 b	732 c
	VHT	334	331	411 a	566 b
G	LT	332	331	661 b	776 e
	VHT	334	330	414 a	571 b
F	LT	331	329	326 e	446 a
	VHT	331	328	146 c	261 d
E	LT	332	331	687 f	717 c
	VHT	335	330	302 d	429 a
F-value	Formulation	-	-	1088	1030
	Drying	-	-	3501	2172
	Interaction[c]	-	-	97	35

[a]Relative standard deviation<0.2%.

[b]Means in the same column with the same letter are not significantly different (p>0.05).

[c]Interaction between drying and formulation.

Results are means of 6 replicates.

Table 4. Essential amino acid scores (EAAS), total essential amino acid (EAA) and dispensable amino acid (DAA) contents in raw material and in low (LT) and very high temperature (VHT) dried pasta. W, wheat; G, Gluten; F, Faba bean; E, Egg

AA	Anses Recommendation (mg/g protein)	EAAS (%, Anses recommendation)[a]											
		Blends of raw material				LT dried pasta				VHT dried pasta			
		W	G	F	E	W	G	F	E	W	G	F	E
His	17	159	155	182	169	141	135	158	159	147	133	172	151
Ile	27	111	112	118	121	109	96	111	103	110	98	94	107
Leu	59	104	107	112	112	108	102	114	107	105	104	106	109
Lys	45	56	51	107	81	46	41	86	64	45	39	74	64
Sulfur AA	23	131	137	104	165	123	147	115	159	149	132	124	140
Aromatic AA	41	161	176	171	208	172	150	147	177	149	159	159	184
Thr	25	90	95	116	116	94	97	120	116	88	95	112	115
Val	27	116	117	127	135	120	119	131	130	115	116	111	137
		AA (mg/g protein)											
EAA		294	303	334	355								
DAA		706	698	666	645								

AA: amino acid, EAA: essential amino acid, DAA: dispensable amino acid, EAAS: essential amino acid score.

His: histidine, Ile: Isoleucine, Leu: Leucine, Lys: Lysine, Thr: Threonine, Val: Valine.

[a]Triptophan amino acid was not analyzed.

Table 5. Results of two-way analysis of variance and an LSD test of the degree of hydrolysis of proteins (°H) by pepsin for 30 min, and 30 min pepsin +180 min pancreatin of cooked to optimal cooking time + 1min wheat (W) pasta, and gluten (G), faba bean (F) and egg (E) enriched-pasta

Protein hydrolysis	Comparison of means (LSD) test[a]								
	Analysis of variance			Effect of formulation				Effect of drying	
	Effects	*F-value*	*p-value*	W	G	F	E	LT	VHT
by pepsin	Formulation	14.3	0.0001	3.82 a	4.14 ab	6.40 c	4.97 b		
	Drying	0.08	0.7862					4.88 a	4.79 a
	Interaction	2.4	0.1031						
by pepsin and pancreatin	Formulation	49.7	0.0000	42.36 a	39.24 b	46.22 c	44.28 d		
	Drying	1.5	0.2432					43.28 a	42.77 a
	Interaction[b]	8.6	0.0013						

[a]Means in the same row with the same letter are not significantly different ($p>0.05$). For each analyzed effect, the mean value for all conditions tested for the other effect is given.

Results are means of three replicates.

Figures:

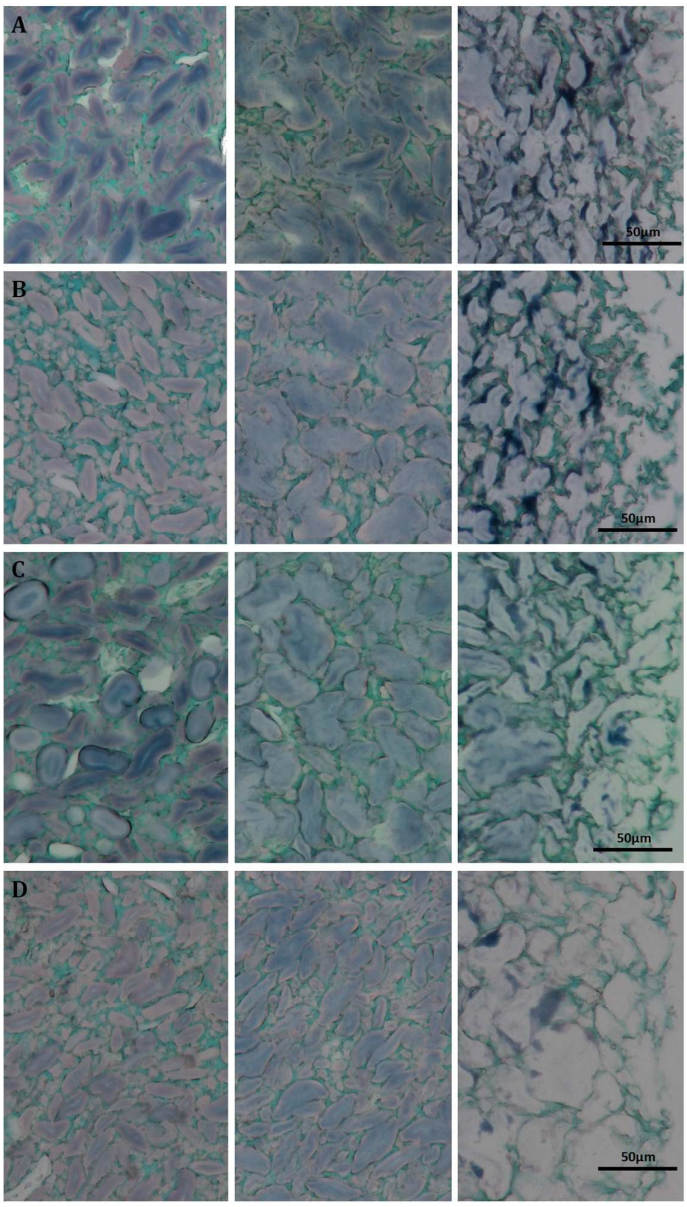


Figure 1 Light microscopy image of low temperature wheat- and protein enriched-pasta cooked to their optimal cooking time +1 min, from the central (on the left) to the external region (on the right). A: Wheat-pasta; B: Gluten-pasta; C: Faba bean-pasta and D: Egg-pasta.

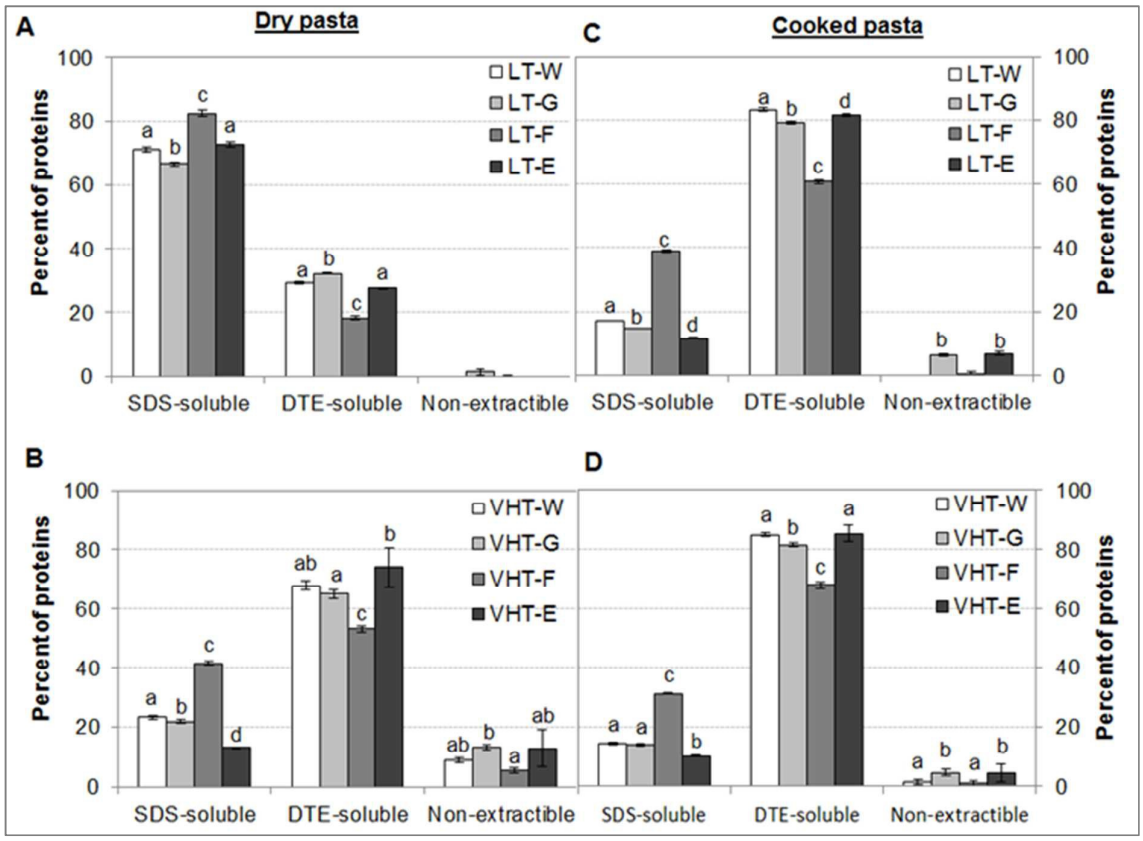


Figure 2 SE-HPLC analyses of soluble proteins in sodium dodecyl sulfate (SDS) and dithioerythritol (DTE) and non-extractable proteins in wheat- (W) pasta and gluten (G), faba bean (F) and egg (E) enriched-pasta, dried at low temperature (LT) (A and C) and very high temperature (VHT) (B and D), in the dry state or after cooking to optimal cooking time +1 min. Bars bearing different letters differ significantly from each other ($p<0.05$). Results are means of 3 replicates.

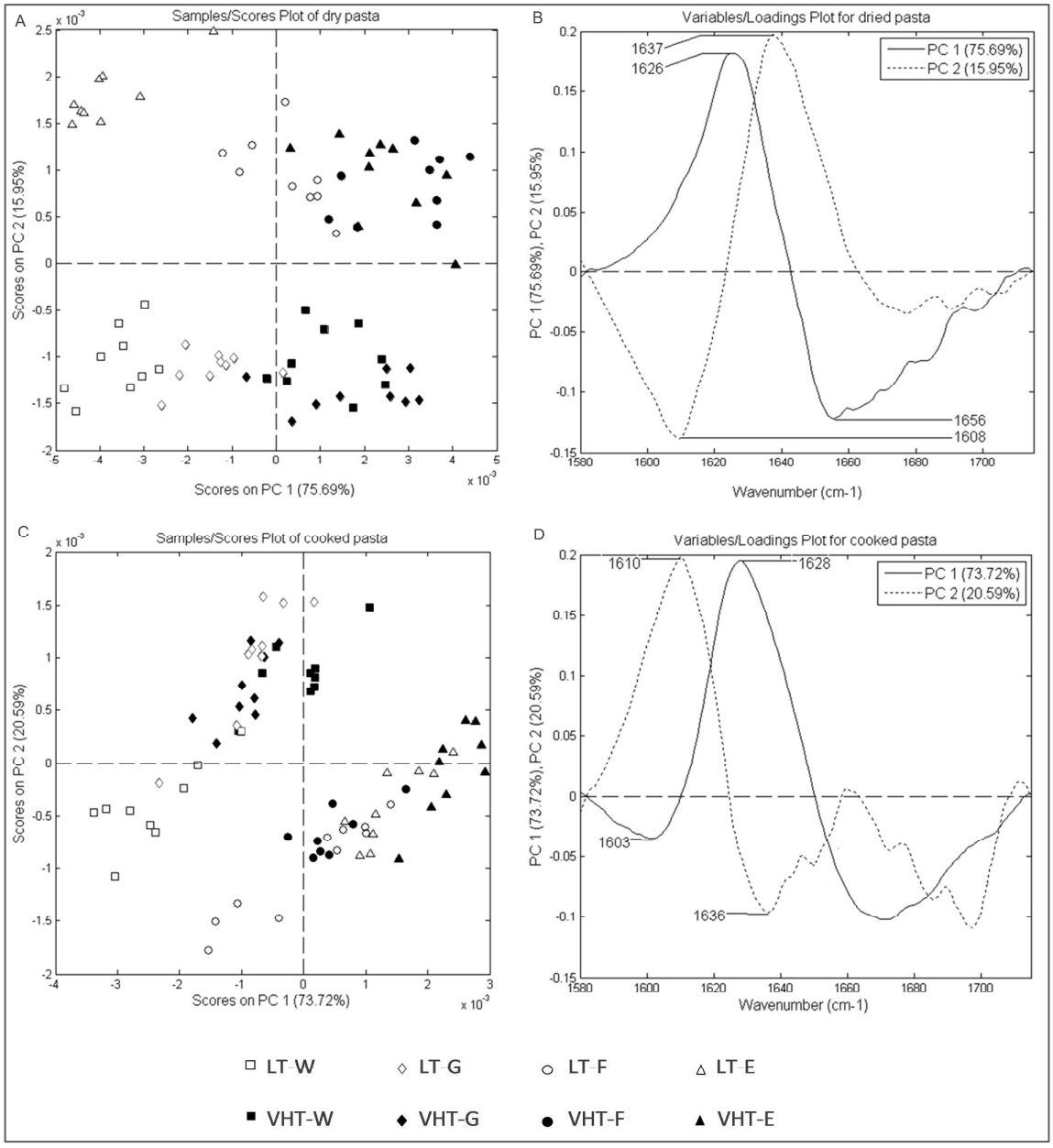
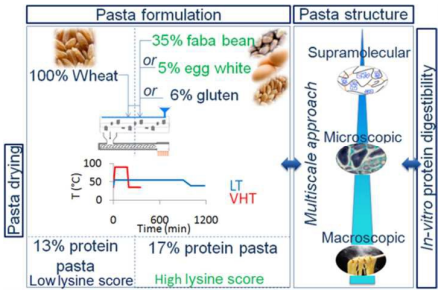


Figure 3 PCA analyses (on the left) and the loadings (on the right) corresponding to spectra of dry (A and B) and cooked at optimal cooking time +1 min (C and D) wheat- (W), gluten- (G), faba bean- (F) and egg- (E) pasta. Spectra were means of 9 replicates of each pasta sample. Analysis were performed in the amid I region (1,580-1,720 cm-1). The two principal components (PC1 and PC2) explained more than 91% of the total variance in both dry and cooked pasta samples.



Highlights

Pasta enrichment with gluten, legume or egg increased its protein content. However, legume pasta had the best amino acid profile and *in-vitro* protein digestibility due to its specific structure.