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Functional, antioxidant and rheological properties of meal from immature durum wheat

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Abstract

Mixtures obtained by the addition to semolina of wholemeal flour derived from durum wheat kernels harvested at the milky phase were analysed for their levels of fructans, vitamin C, glutathione and for their rheological characteristics to evaluate the possibility of obtaining wholemeal with increased nutritional value for preparing functional foods, without addition of exogenous additives or antioxidants. Wholemeal from immature kernels (WIK) contained almost three times more fructans than semolina from mature kernels (SMK). Moreover, wholemeal from immature kernels contained much higher amounts of ascorbate and glutathione than SMK. Consistently, wholemeal from immature kernels also had higher total antioxidant capability than SMK. The addition of a certain percentage of WIK to SMK conferred an increased tenacity to the dough. The role of the different biochemical properties, mainly related to the ascorbate redox enzymes, of WIK versus SMK in determining the rheological properties of the mixtures is discussed.

Keywords: Antioxidant; Fructooligosaccharides; Immature kernels; Ascorbate redox enzymes

1. Introduction

Durum wheat is the preferred raw material for different products (i.e. pasta). Many factors influence the quality of durum wheat and its products, but there is universal agreement that protein content and gluten strength are the most important. The functional properties of wheat proteins depend on the composition of their constituent polypeptides, their molecular properties and interactions with one another and other flour constituents (Bushuk and McRitchie, 1988). The gluten proteins are characterised by strong aggregation tendencies resulting from their hydrogen bonding potential (Wrigley and Bietz, 1988). Their solubility and inter-protein bonding are probably the main factors responsible for the unique visco-elastic properties of wheat gluten. Disulfide bonds, hydrogen bonds and hydrophobic interactions are considered the most important associative forces in gluten structure. Modifications of thiol and disulfide groups have been extensively used as a means of improving dough during mixing and as evidence of the contribution of these groups to dough properties. During grain ripening important changes in protein composition occur; albumins and globulins decrease in the early weeks after anthesis but remain relatively constant from the fourth week through to maturity (Bollini et al., 1981). Wheat albumins and globulins include some enzymes, so enzymatic activities also change during ripening and this also affects the level of disulfide bonds in isolated gluten (Schofield and Booth, 1983).

Recently, research and consumer interests have focused on a more integrated approach to the nutritional value and health implications of foods. In this context, the fructooligosaccharides (FOS), fructose polymers with different degree of polymerisation have received much attention. These naturally occurring oligosaccharides are synthesized and accumulated in many plants and have beneficial effects as food ingredients

Abbreviations: AFR, ascorbate free radical (also known as monodehydroascorbate or ascorbyl); AFRR, ascorbate free radical reductase; ASC, ascorbate; AOX, ascorbate oxidase; APX, ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; DTT, dithiothreitol; FOS, fructooligosaccharides; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; SMK, semolina from mature kernels; WIK, wholemeal from immature kernels.

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(Ritsema and Smeekensy, 2003). Their beneficial effects arise through stimulation of the growth of bifidobacteria in the human colon, by suppressing putrefactive pathogens, rebalancing metabolic activities and improving the bioavailability of nutrients (Gibson and Roberfroid, 1995; Hidaka et al., 1991; Tomomatsu, 1994). The physicochemical properties, sweetening power and low caloric value of FOS make them suitable for addition to pastry, confectionery and dairy products. Fructan metabolism is now understood at the molecular level and fructan synthesis and breakdown can be adapted for the use in human foods (Ritsema and Smeekensy, 2003). Large quantities of FOS are stored in the stems and grains of wheat for much of its growing cycle (D'Egidio et al., 1997). Maximum accumulation of FOS in wheat kernels occurs between the second and third week after flowering at the physiological stage termed as the milky phase, thereafter they rapidly decrease. The high level of fructans in immature wheat grains seems particularly encouraging for the utilisation of the kernels harvested at the milky phase as a functional food. At this ripening stage, the storage proteins which give strength to flour begin to be synthesised in the kernels. Gliadins have been detected at 10 days after anthesis while high-molecular weight glutenin subunits (HMW-GS) and low-molecular weight glutenin subunits at 13 days after anthesis; and these proteins continue to accumulate in the kernels until physiological maturity is reached (Mujoo and Ng, 2003).

Other metabolites conferring 'healthy' properties on foods are the so-called antioxidants. Among these, vitamin C also affects the rheological properties of flour, apart from its well known nutritional value. It has been reported that the addition of vitamin C in the dough improves the kneading (Melville and Shattock, 1938; Nakamura and Kurata, 1997). The presence of dehydroascorbate (DHA), the oxidised form of ascorbate (ASC), more than ASC itself, seems to be responsible for improving the rheological properties, since, due to its oxidising character, it increases the formation of intra- or inter-molecular disulphide bridges between cysteine residues in gluten. Cereal grains are reported not to contain vitamin C (Carnovale and Marletta, 2000), although other analyses show low amounts of DHA in mature kernels (Cakmak et al., 1993; De Gara et al., 1997; 2003). Moreover, at the beginning of their development, wheat kernels contain a considerable amount of ascorbate, apart from a certain amount of DHA. During kernel development and maturation, the vitamin C content decreases from values of about 1 mg g^{-1} dry weight at the milky phase to about 30 μg g^{-1} dry weight at the commercial maturation stage (data derived from De Gara et al., 2003). Wheat kernels possess enzymes responsible for the oxido-reduction of ASC and its oxidised forms, i.e. the ascorbate free radical (AFR) and DHA. These enzymes are: ascorbate oxidase (AOX), which oxidises ASC with concomitant reduction of di-oxygen to water; ascorbate peroxidase (APX), a key enzyme for H_2O_2 scavengers in plant cells; AFR reductase (AFRR) and DHA reductase (DHAR), which are involved in the recycling of ASC by its oxidised forms. DHA can also be reduced to ASC by protein disulphide isomerases (Wells and Xu, 1994). The latter enzyme plays a crucial role in both protein maturation during kernel development and gluten formation, since it catalyses the formation of inter- or intra-molecular disulfide bridges between specific cysteine residues.

We have previously reported that during kernel maturation, remarkable changes also occur in the enzymes responsible for vitamin C oxido-reduction. In particular, immature kernels have APX and AOX activity higher than mature kernels. On the other hand, the activity of DHAR is lower in immature than in mature kernels (De Gara et al., 2003; Every, 1999). Glutathione, another redox metabolite with antioxidant properties, also decreases during kernel maturation, although the mature kernels still have a much larger glutathione pool than vitamin C (De Gara et al., 2003).

With the aim of investigating the possibility of obtaining wholemeal with interesting rheological and nutritional characteristics, without the addition of exogenous additives or antioxidants, different proportions of wholemeal obtained from kernels harvested at the milky phase (Wholemeal from Immature Kernels—WIK) were added to semolina obtained from kernels collected at commercial maturity (Semolina from Mature Kernels—SMK), and the levels of fructans, vitamin C and glutathione, as well as the enzymes of the ASC and GSH oxido-reduction system were analysed.

2. Experimental

2.1. Plant material and milling procedures

Durum wheat, cultivar Simeto, was grown in the experimental field of the 'Istituto Sperimentale per la Cerealicoltura' in Rome. The different development stages of plants were followed to define the ear emergence and flowering time, the latter considered as a reference for the harvesting time. The samples were collected 17 days after flowering and at commercial maturity.

Immature kernels harvested at the milky phase (17 days after anthesis, when the dry weight of the kernels is about 30%) were dehydrated by forced air at 40 °C until at ~10% moisture. Immature kernels were ground in a laboratory mill (FRITSCH Pulverisette type F 14002) to produce a meal. Different proportions of the meal were added to semolina from mature kernels of the same cultivar to evaluate the nutritional and rheological properties of the blends. Mature durum wheat kernels were ground in a pilot plant mill (MLU 202 Bühler) to obtain semolina.

2.2. Determination of metabolites and antioxidant properties

The analysis of soluble carbohydrates was performed after preliminary extraction with 96% v/v ethanol for 1 h at 80 °C, followed by a water extraction for 2 h at 105 °C. The ethanol soluble fraction contained mainly low molecular weight carbohydrates (e.g. mono- and disaccharides), while the water-soluble fraction contained higher molecular weight carbohydrates (e.g. fructans and oligosaccharides); the latter fraction was used for fructans analysis. Glucose and fructose contents were determined in both fractions using enzymatic (glucose oxidase/peroxidase kit by Megazyme method GLC

6/798—Megazyme International Ireland Ltd, Bray, Ireland) or chemical (resorcinol–HCl) (Virgona and Barlow, 1991) procedures respectively. Since fructans consist exclusively of fructose and glucose, their quantification in the water soluble fraction allowed the measurement of the total content of fructans.

Vitamin C (ascorbate plus dehydroascorbate) contents were analyzed in the WIK and SMK according to de Pinto et al. (1999) with some modifications. Briefly, WIK and SMK were homogenized with two volumes of cold 5% meta-phosphoric acid at 4 °C. The homogenate was centrifuged at 20,000 *g* for 15 min at 4 °C, and the supernatant was collected for analysis. Total ascorbate was determined after reduction of dehydroascorbate to ascorbate with dithiothreitol (DTT); and the concentration of dehydroascorbate estimated from the difference between the total vitamin C pool (ascorbate + dehydroascorbate) and ascorbate. The reaction mixture for the assay of the total ascorbate pool contained a 0.1 ml of the supernatant, 0.25 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 0.05 ml of 10 mM DTT. After incubation for 10 min at room temperature, 0.05 ml of 0.5% *N*-ethylmaleimide was added to remove excess DTT. Ascorbate was determined in a similar reaction mixture except that 0.1 ml H_2O was added rather than DTT and *N*-ethylmaleimide. Colour developed in both reaction mixtures after addition of the following reagents: 0.15 ml of 10% trichloroacetic acid, 0.2 ml of 44% *ortho*-phosphoric acid, 0.2 ml of 4% α,α'-dipyridyl in 70% ethanol and 0.3% (w/v) $FeCl_3$. After mixing on a vortex mixer, the mixture was incubated at 40 °C for 40 min and the absorbance read at 525 nm. To avoid interference due to the presence in the kernels of compound other than ascorbate able to react with α, α'-dipyridyl, a second blank was used for each sample without DTT and in which purified AOX (2U of *Cucurbita sp*. AOX, Sigma) was added before the 10 min incubation step. The addition of AOX induced complete oxidation of the ascorbate present in the sample. The absorbance values obtained for these blanks were not due to the presence of ascorbate and were subtracted from the values obtained for the samples used for the total vitamin C determination. A standard curve was developed based on ascorbate in the range 0–50 μg ml^{-1}.

The glutathione pool was assayed on the same extracts as vitamin C according to de Pinto et al. (1999).

Total antioxidant activity was evaluated according to Brand-Williams et al. (1995) by measuring the ability of the extracts to reduce 2,2-diphenyl-1-picrylhydrazyl radical (DPPH).

2.3. Enzyme assays

SMK or WIK (1 g) was mixed vigorously at 4 °C with an extraction buffer (Tris–HCl 50 mM, pH 7.4, 1:2 w/v), using a ceramic pestle and mortar. The homogenate was centrifuged at 18,000*g* for 15 min and the supernatant was passed through a Sephadex G25 column and used for enzyme assays.

AOX (L-ascorbate: oxygen oxidoreductase, EC 1.10.3.3) was assayed by measuring the oxidation rate of ASC at 265 nm in a reaction mixture consisting of 50 μM ASC, 50–100 μg protein and 0.1 M phosphate buffer, pH 6.4.

APX (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) was assayed by following the H_2O_2-dependent oxidation of ASC at 265 nm in a reaction mixture containing 0.1 M Tris–acetate buffer, pH 6.4, 50 μM ASC, 170 μM H_2O_2, 50–100 μg protein. The non-enzymatic H_2O_2-dependent oxidation of ASC, as well as the oxidation of ASC not dependent on H_2O_2 addition was subtracted.

DHAR (glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1) and AFRR (NADH: ascorbate free radical oxidoreductase, EC 1.6.5.4) were assayed as described in de Pinto et al. (2003). GR (NADPH: glutathione disulfide oxidoreductase, EC 1.6.4.2) was assayed by the method of Osswald et al. (1992).

Protein content was measured according to Bradford (1976) using bovine serum albumin as a standard. Enzyme activities were measured using a Beckman (Fullerton-CA) DU 7000 spectrophotometer. Native polyacrylamide gel electrophoresis (PAGE) for DHAR was performed as described by De Gara et al. (1997).

2.4. Rheological properties

The rheological characteristics of the semolina and of the blends with meal from immature durum wheat were evaluated using a Chopin alveograph complete of Alvedink according to standard procedures for durum wheat. The dough was mixed for 4 min, and after a rest of 18 min, mixed again for 4 min.

3. Results and discussion

Wholemeals were obtained from kernels collected at the milky phase (17 days after anthesis), when the kernels had about 30% dry weight. At this stage, kernels still have levels of FOS and vitamin C almost 5–10- and 10–15- times higher, respectively, than mature kernels, when these metabolites are expressed on a dry weight basis (De Gara et al., 2003; Paradiso et al., 2003). Although, kernels collected at an earlier phase (9–13 days after anthesis) have a higher level of fructans than that of the immature kernels harvested at the milky stage (Nardi et al., 2003), but technical problems due to their excessively reduced size and weight precludes their use. To obtain wholemeal useful for functional food preparation, different proportions of WIK were mixed with the SMK. When wholemeal and semolina were prepared from kernels obtained from a field crop, the level of fructans was 55 mg/g dry weight in WIK and about 20 mg/g dry weight in SMK (Fig. 1A). These values are in the range of variability due to the agroclimatic conditions (D'Egidio et al., 1993) and, in spite of being lower than those from experimental trials (De Gara et al., 2003; Nardi et al., 2003), the relative differences are similar. As expected, the FOS content of the mixtures was proportional to the amount of WIK added (Fig. 1A).

The amounts of vitamin C in WIK, SMK and in the different blends are reported in Fig. 1B. The amount of vitamin C (ASC + DHA) detectable in WIK was much lower than that

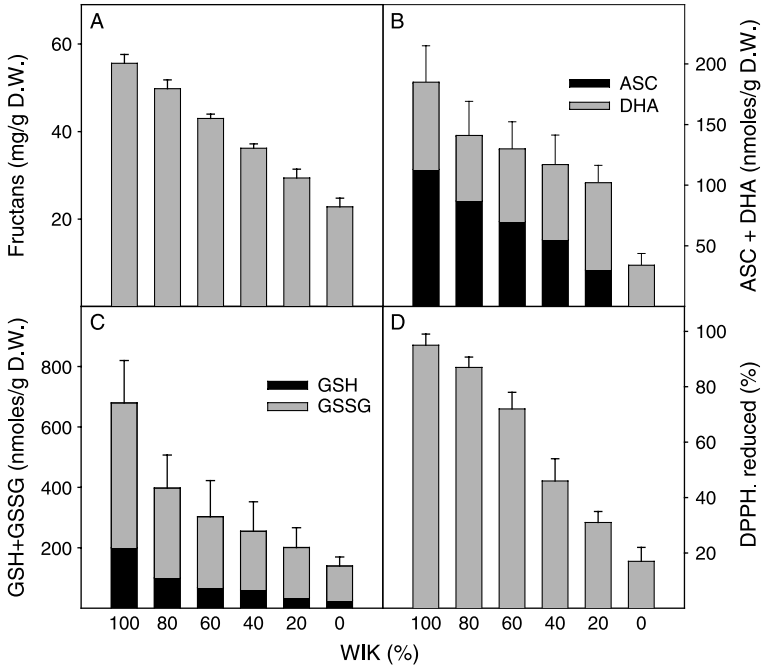

Fig. 1. Contents of fructans, ascorbate, glutathione and antioxidant global capability of WIK, SMK and mixtures containing different WIK percentages. The values represent the mean of four independent experiments ± SD.

reported for kernels analysed immediately after harvesting (De Gara et al., 2003; Paradiso et al., 2003). This was probably a consequence of the dehydration process required for storing and milling the immature kernels. However, in spite of this, WIK still contained more vitamin C than SMK (Fig. 1B). About 60% of the vitamin C was still present as ASC in WIK while SMK contained only a low amount of DHA as the unique form of vitamin C. In the blends, the vitamin C content increased proportionally to the amount of WIK added (Fig. 1B). The level of glutathione pool (glutathione-GSH-plus glutathione disulfide-GSSG-) was also higher in WIK than in SMK and values decreased as the percentage of the WIK in the mixtures decreased (Fig. 1C). GSH was present both in SMK and WIK, although as the minor component of the glutathione pool.

ASC and GSH are not the only antioxidant molecules present in the kernels; therefore, the global antioxidant capability of WIK and SMK, as well as of the mixtures, was

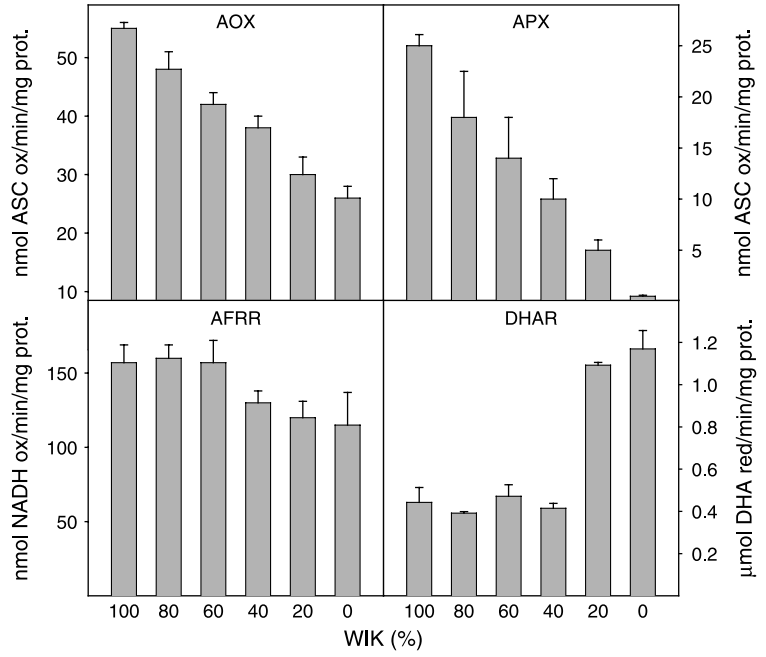

Fig. 2. Activity of the ASC redox enzymes in WIK, SKM and mixtures containing different WIK percentages. The values represent the mean of four independent experiments ± SD.

determined. As shown in Fig. 1D, the total antioxidant capability of WIK was almost five times higher than that of SMK. As expected, the antioxidant capability of the mixtures depended on the relative proportion of WIK and SMK.

The reduced and oxidised forms of both vitamin C and glutathione can be interconverted by specific redox enzymes which are responsible for the redox state of ASC/DHA and GSH/GSSG present in the cells. Since, the reduced and oxidised forms of these two redox pairs affect the rheological properties of the dough in different ways, DHA and GSSG having a higher capability to increase dough tenacity than their reduced forms (Kaid et al., 1997; Nakamura and Kurata, 1997), the presence of active ASC and GSH redox enzymes in the dough could also be relevant for changing its rheological properties. For this reason, the activities of the enzymes involved in the ascorbate and glutathione oxido-reduction were determined both in WIK, SMK and in their blends.

As expected, the processes required for kernel dehydration and milling led to a decrease in the activities of all the enzymes assayed with respect to those detected in the kernels analysed immediately after harvesting (De Gara et al., 2003). However, the ASC–GSH redox enzymes were still active both in WIK and SMK (Figs. 2–4). The activities of the enzymes were quite different in WIK and SMK, and were generally in agreement with the differences shown during kernel maturation (De Gara et al., 2003). WIK had a higher ASC oxidizing capability than SMK, because both AOX and APX were more active in WIK than in SKM. Intermediate values were observed in the blends (Fig. 2). In respect to AOX, the data are in agreement with previous results (Every, 1999). Since AOX requires only molecular oxygen for the oxidation of ASC, its activity depends only on the presence of ASC in the dough. It is noteworthy that increased AOX activity could also contribute to the anaerobic condition required for fermented food preparation. It is probable that the relevance of APX for DHA generation in doughs is much lower than that of AOX, since APX requires H_2O_2 as co-substrate but H_2O_2 production has not been reported in doughs. AFRR had only a slightly higher activity in WIK than in SMK (Fig. 2). On the other hand, DHAR had a higher activity in SMK than in WIK (Fig. 2). Surprisingly, DHAR activity was not proportional to the relative amounts of WIK and SMK in the blends; but, in the range of WIK addition between 80 and 40%, it maintained values very similar to that of WIK alone. However, with addition of 20% WIK to SMK the DHAR activity was similar to that of SMK alone (Fig. 2). This unexpected behaviour of DHAR could be explained by the presence in WIK of some inhibitor affecting its activity. Consistently, native PAGE of DHAR showed the presence of four proteins with DHA reducing activity in SMK; these were still evident in the mixture containing only 20% of WIK; whereas only two bands were detectable in WIK and in the mixtures containing proportions of WIK higher than 20% (Fig. 3). This result supports the hypothesis that some metabolites present in WIK extract could affect the activity of the two DHA reducing proteins which are active only in SMK and when the percentage of WIK in the mixture is low.

As found for DHAR, glutathione reductase (GR) also had a higher activity in SMK than in WIK, but in the mixtures it had values proportional to the relative percentages of WIK and SMK composing them (Fig. 4). A relationship between DHAR and GR was expected, since these two enzymes work co-ordinately in the ascorbate-glutathione cycle to reduce DHA (Noctor and Foyer, 1998).

The presence of WIK in the dough strongly affected the rheological properties of SMK by increasing dough tenacity (Fig. 5). Its effect was so strong that the alveographic test was only possible at the lower proportions of meals from the immature kernels (20–40%). The increase in dough tenacity induced by WIK is probably due to the increased level of antioxidants during gluten formation, in particular to the increased ascorbate. This was confirmed by the addition to SMK of vitamin C, at the same concentration found in WIK. This induced an alteration of dough rheological properties with a trend similar to that caused by WIK addition (increase in *P*/*L*

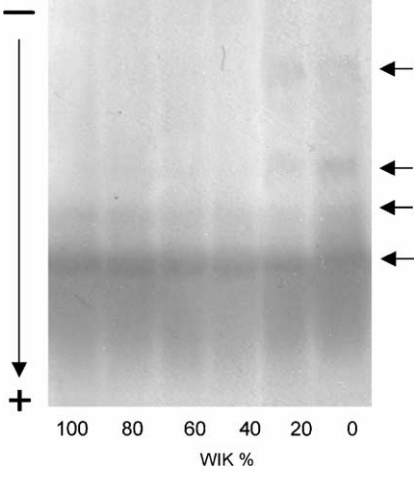

Fig. 3. A representative native PAGE of DHAR in WIK, SMK and mixtures containing different WIK percentages. 200 μg proteins were loaded in each lane.

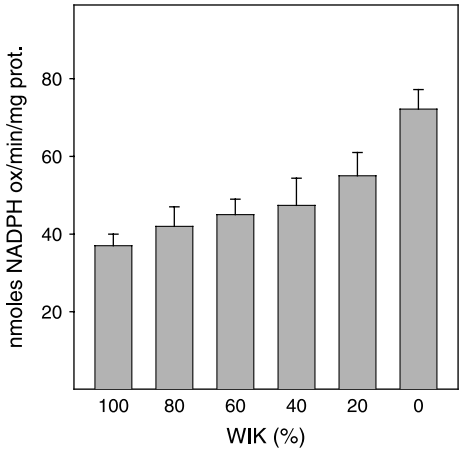

Fig. 4. GR activity in WIK, SKM and mixtures containing different WIK percentages. The values represent the mean of four independent experiments ± SD.

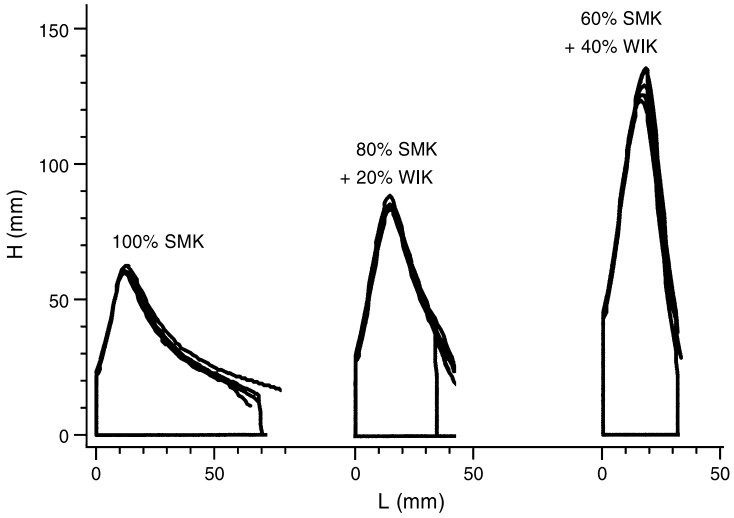

Fig. 5. Alveographic profiles of durum wheat semolina and relative blendings with immature meals. The values of *W* were 126, 118, 148 for 100% SMK, 80% SMK +20%WIK, and 60% SMK+40% WIK, respectively, and they did not significantly changed. Statistically significant differences were evident for the *P*/*L* values of the different blends (1.2; 3.4 and 5.8 for 100% SMK; 80% SMK+20% WIK; and 60% SMK+40% WIK, respectively).

values from 1.2 to about 3 without significant changes in *W* values). The effect of vitamin C in increasing dough tenacity has been reported previously (Melville and Shattock, 1938; Nakamura and Kurata, 1997). The addition of glutathione also affected dough rheological properties, but it also altered the *W* value, in addition to *P*/*L* ratio (data not shown). It may be noted that WIK is not only a source of antioxidant metabolites, but also has different both quantitative and qualitative enzyme activities. The different quantitative of the ASC redox enzymes in the blend could also be critical in conferring rheological properties different to those induced by the simple addition of the various metabolites. It is worth noting that the higher ASC oxidising capability, conferred on the dough by the presence of WIK, increases DHA formation. At the same time, the presence of WIK determines a reduction in the DHA reducing capability of the dough. These two events increase the availability of DHA for the formation of the intra- and inter-molecular disulphide bonds, thus contributing to increased dough tenacity. Moreover, the different amount of fructans, molecules with high hydrophilic properties, could also contribute to the alteration of the alveograph profiles of the dough by acting as a dilution factor in gluten network formation.

4. Conclusion

The results indicate that the addition of a certain proportion of WIK to SMK could be of relevance for the preparation of functional foods naturally enriched in FOS. The addition of WIK could also be very useful for correcting doughs with low tenacity. This activity emphasizes the need to take into account the changes in the rheological properties of the dough induced by WIK addition, which are partly explained by the different biochemical properties of the wholemeals, if immature kernels are to be used for wholemeal preparation.

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