Influence of carob flour ingredients on wheat-based systems

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Abstract

Carob flour and its main constituents have been shown to possess nutritional benefits and might be considered as low-cost competitor to other food ingredients in enriched food products.

In this study, we performed a DSC characterization of the thermal properties of carob flour and derived fractions (carob protein fraction and locust bean gum) at various moisture percentages, aiming at understanding their behaviour in more complex matrices. Furthermore, wheat/carob ingredient blends were investigated at different moisture content and components ratios to asses and dissect the interplay between carob and wheat flour macromolecules following their thermal transitions.

The results indicated that only the carob protein fraction is adequate as ingredient for enriched wheat-based baking products since it does not significantly influence the water partition between the starchy and protein phases. Such a prediction was confirmed by technological trials, *i.e.*, by preparing and comparing reference wheat loafs and ones enriched with 5% w/w of carob protein.

1. Introduction

Carob (*Ceratonia siliqua L.*) is a leguminous shrub native from the Mediterranean region, mainly from Spain, Italy, Greece, Portugal and Morocco (Dakia et al., 2007) and it was, in a not far past, basic food source selected by human communities because of its nutritional properties.

Carob seeds are extremely tough and consist of coat (30-33%) rich in antioxidants, endosperm (42-46%), and germ (23-25 %), and, nowadays, carob seed processing is mainly conducted to yield the corresponding endosperm either by chemical or by thermo-mechanical treatment (Salinas et al., 2015). Indeed, carob seed endosperm is a source of locust bean gum, also called carob gum or E410, which is a non-ionic galactomannan vegetable gum chiefly consisting of high-molecular-weight hydrocolloidal polysaccharides composed of galactose and mannose units in an average ratio of 1:4, respectively (Pegg, 2012). Locust bean gum founds its application in food industry since it is able to form synergic gels with other hydrocolloids at low temperature, significantly improving gel strength and texture and preventing syneresis (Kulkarni and Shaw, 2016).

The residue of the milled seed is considered as a by-product in this process and is generally underutilized in the food industry. As a matter of fact, carob germ can be recovered and, after milling and heat treatment (Salinas et al., 2015), carob germ flour can be used as dietetic human food (Dakia et al., 2007), in the nutrition of sportspeople (Bengoechea et al., 2008) or as a potential ingredient in cereal-derived foods for celiac people (Tsatsaragkou et al., 2014) for its specific nutritional properties. Indeed, carob germ flour, also called as “caroubin”, is isolated from carob bean embryo and has been found to contain about 32% albumin and globulin and about 68% glutelin, with no prolamins detected, and with only 5% of insoluble protein fraction (Smith et al., 2010). Literature reports values around 50% for protein content with high amount of lysine, arginine and glutamic acid for carob germ defatted flour (Maza et al., 1989), that is higher than those observed for other beans, such as pea (18.83%) and soybean (34.35%) (Marcone et al., 1998). Hence, carob germ flour could be considered a low-cost competitor to other food proteins like dairy or soy proteins (Bengoechea et al., 2008), with a high potential as a food ingredient (Tsatsaragkou et al., 2012).

Carob germ flour was first described for its use in the production of wheat-free pasta and baked products in a 1935 patent (Smith et al., 2010), and, since then, several other studies have been conducted on carob flours to ameliorate the properties of food products added up with carob ingredients.

The supplementation of cereal flours with carob ingredients is highly considered as a valuable mean to improve the nutritional quality of food products such as those based on wheat flour, which is poor in some essential amino acids like lysine and threonine (Salinas et al., 2015). Moreover, wheat flour often suffers from the reduction of relevant amounts of minerals and vitamins as a consequence of extended grain milling process, and the incorporation of carob flour within cereal-based products may lead to a significant increase of minerals (Fe, Ca, Na, K, P and S), vitamins (E, D, C, Niacin, B6 and folic acid) and antioxidant compounds (such as phenolic compounds) (Salinas et al., 2015), though resulting in a decreased digestibility

of starch and proteins likely due to a different nutrient susceptibility to hydrolysis, as shown in fortified durum wheat pasta (Sęczyk et al., 2016).

On the other hand, blending wheat flour with carob flour modifies the rheological properties of doughs, as well as the characteristics of the final baked good. Turfani et al. (Turfani et al., 2017) showed that the addition of carob flour to wheat bread generally leads to tenacious, stable and more water-demanding doughs, contrary to what usually happens as a consequence of the addition of other legume flours (*e.g.*, lentil flour), which may reduce dough tenacity, extensibility and strength. Instead, the development time of carob fortified bread dough is extended, similarly to other legume flours such as lentil and pea flours (Kotsiou et al., 2021). In any case, wheat/carob blends influence the technological performances of loafs depending on the amount of carob flour, with threshold-depending positive/negative effects (Turfani et al., 2017).

As a matter of fact, the inclusion of an additional ingredient such as carob flour as it is or as fractions in cereal-based matrices may lead to technological aspects difficult to predict. Furthermore, such difficulties in predicting the characteristic of the final baked product are also amplified by the relevant variability in both the amount and the physicochemical properties of carob seed constituents depending on the carob genotype used for the production of carob flour as a whole and/or as fractions. Indeed, literature reports that seeds from different carob tree populations (different varieties and growth locations) show different ratios between the protein and the polysaccharide fractions, as well as different amounts of the main monosaccharide units (especially galactose and mannose) present in the locust bean gum portion, and all these peculiarities are responsible for different viscosity behaviours (Rizzo et al., 2004). Consequently, this complex scenario often induces researchers to investigate products basing on the phenomenology and on a trial-and-error approach as regards the product formulation (Tsatsaragkou et al., 2014).

In order to rationalize the influence of carob flour ingredients on the technological properties of the wheat-based systems, it should be considered that the behaviour of a dough is directly related to the role played by the macromolecules. In particular, a dough is a heterogeneous system, since starch carbohydrates and flour proteins are thermodynamically incompatible biopolymers, hence the macromolecules included in a dough induce phase separation and govern the water partition (Tolstoguzov, 1997). Accordingly, water is distributed among separate aqueous phases, each of which is richer in a given polymer with respect to the nominal dough composition (Fessas et al., 2008; Fessas and Schiraldi, 2001, 1998). Literature reports that the water partition and the inter-phase interactions are dominant and dictate the formation of the macroscopic structure and the texture of the final baked product (Fessas et al., 2008). The addition of a new component, protein and/or polysaccharide, may influence this water phase separation and, in turn, the technological properties of the system (starch gelatinization, gluten network, *etc.*).

In this context, to the best of our knowledge, a systematic investigation of the effects of carob flour as it is and as fractions on wheat dough aimed at pointing out the interplay between all the constituent

biopolymers is still missing, especially considering that a high affinity of locust bean gum for water is known from the literature (Torres et al., 2012) and it is expected to somehow influence the water distribution among phases.

To address this point, calorimetric approaches are highly suitable for analysing the formation of baking food structures, basing on the study of the interplay of matrix phases, and would help to reveal the physics underlying the properties of wheat/carob blends, discriminating the components contribution as regards starch gelatinization, protein behaviour and water partition.

To this aim, in this work, the thermal properties of carob flour as it is (CF) and its components, *i.e.*, carob germ flour (from now on referred to as carob protein fraction, CP, for the sake of simplicity) and locust bean gum (LBG), separately and in mixtures with wheat flour, were investigated through differential scanning calorimetry (DSC) focusing on the technological aspects related to enriched bread new formulations. Samples with several components ratios and moisture contents were considered. A validation of the technological prediction emerged from this study was also performed in a real system and the technological properties of enriched wheat bread were compared to the reference.

2. Materials and Methods

2.1. Materials

Carob flour (CF) and Carob germ flour (or Carob protein fraction, CP) were provided by Cypriots local producers. CF was obtained from carob seed (germ, endosperm (LBG) and coat) with moisture content 9.4%, protein 23.0%, dietary fibre (including LBG) 51.8% and lipid 1.8%. Instead, CP was with moisture content 8.0%, protein 64.1%, dietary fiber (including LBG) 13.3% and lipid 10.2%.

Wheat flour (W) was commercial and characterized by moisture content 12.3%, protein 9.1% and lipid 2.0%.

Locust Bean Gum (LBG) was obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany).

2.2. Differential Scanning Calorimetry

Thermal properties of different flours were analysed through a PerkinElmer DSC6 (PerkinElmer, Waltham, MA, USA) working with stainless steel sealed pans throughout a temperature range from 10°C to 150°C at 2°C·min^{-1} scanning rate. An empty pan was used as reference and calibration was carried out with indium as standard. Samples were prepared by directly adding an adequate amount of distilled water within the pans containing about 15 mg of sample (powder) to achieve overall moistures of 30%, 40%, 55% and 70%. The real final water content for each sample was assessed after the DSC analysis by piercing the cold pans and by desiccating them at 105°C.

Data were analysed with the dedicated software IFESTOS following procedures reported in previous studies (Bresciani et al., 2022; Emide et al., 2023). In brief, the excess heat capacity $C_P^{exc}(T)$ / J·K^{-1}·g$^{-1}_{\text{dry matter}}$, *i.e.*, the

difference between the apparent heat capacity $C_P(T)$ of the sample and the baseline, was recorded across the scanned temperature range. The baseline for each measurement was obtained by considering the respective second heating scan trend as reference, as described in the literature (Fessas and Schiraldi, 2000). The peak integration performed after the baseline subtraction corresponded to the overall enthalpy of the transition expressed in terms of dry sample. Three replicates were performed for each sample and one representative curve for each sample was reported.

2.3. Bread making

For bread preparation, doughs were prepared by mixing wheat flour with the following materials: tap water, moist yeast (L'hirondelle, S.I. Lesaffre, France), sugar, salt (iodised sea salt, Kallasgroup S.A., Katerini Branch, Greece) and shortening (Vitam, Unilever S.A, Athens, Greece). The basic recipe for the dough (based on the weight of the flour) was 4% yeast, 3.5% shortening (margarine), 3% sugar and 2% salt. Compared to the reference systems (simple wheat dough), wheat doughs also including carob protein at 5% w/w were prepared. The amount of water added to doughs was 55% (based on the weight of the flour). The dry ingredients (wheat flour, carob protein, sugar, salt) were first mixed in a mixer (Hobart N50, Hobart Co., Troy, OH, USA). Then, the yeast mixed with the appropriate water amount was added progressively, followed by the addition of melted shortening to the final blend. All the ingredients were mixed for 5 min using a mixer at a speed of 475 rpm. After complete mixing, 420 g of dough was placed in oiled pans (20 x 10 x 6 cm) and was fermented at 35^{o}C and 85% RH for 50 min. Following fermentation, samples were baked at 180^{o}C for 45 min in a convection oven. The loaves were cooled to room temperature, to ensure that condensation does not form on the inside of the package and placed in polyethylene bags for 24 h before determination of their physical properties. Breads were made in duplicate.

2.4. Bread analysis

2.4.1.Bread moisture

The evaluation of moisture in the bread was performed using AOAC method 935.36.

2.4.2.Crumb texture

The firmness of bread crumb was estimated with the 74-09 method of the American Association of Cereal Chemists (2000) in an Instron (Universal Testing Machine, Model 1100, Massachusetts, USA) equipped with a 50 N load cell. The crumb texture determination was done according to Tsatsaragkou et al. (Tsatsaragkou et al., 2012): a slice of 2.5 cm (thickness) from the centre of the loaf was compressed to 40% of its initial height with a 4 cm diameter probe coming down with a speed of 101 mm/s. The force (N) reading, measured at 40% of compression, expressed the resistance of the crumb to the penetrating probe and

represented the crumb firmness. The crumb firmness was measured in duplicate after 24 h of bread preparation.

The crumb's relative elasticity testing was carried out in crumb cubes of 2 x 2 x 2 cm (length x width x height). The cubes were always cut from the same, almost middle part, of the loaf. The measurements were done in triplicate. A uniaxial compression test with subsequent relaxation phase that lasted 4 min was applied in order to determine the textural and viscoelastic properties of the bread crumb. Crumb's relative elasticity was calculated at 25% compression, within the linear viscoelastic region.

2.4.3.Porosity determination and crumb grain measurements

For porosity measurements, samples of 1.5 x 1.5 x 1.5cm (length x width x height) from the geometric centre of the crumb were taken for all breads. The volume of solids (V_s, m^3) was measured with gas pycnometer (Stereopycnometer SPY-3, Quantachrome, Syosset, N.Y., USA), where helium was used as the displacement gas.

For each measurement, three different samples were used, each measured three times, using the equations referred in the study of Tsatsaragkou et al. (Tsatsaragkou et al., 2012).

Two slices of 1.5 cm thickness from each of the 2 breads were cut, so that four slices were used in total for each case. Images of the slices were captured using a flatbed scanner (HP scanjet 4370, HewlettePackard, USA). Image analysis of bread slices was carried out using Image analysis software (ImageProPlus 7, Media Cybernetics, USA). Values of scanned images were obtained in pixels and converted into cm by using known length values. Average cell diameter and measured cells/cm were determined.

2.4.4.Statistical analysis

Analysis of variance (ANOVA) was performed using STATGRAPHICS (Centurion XV.II.). LSD test was used for comparison of sample data, and evaluations were based on a significance level of $p < 0.05$.

3. Results and Discussion

3.1. DSC Analysis of carob flour and its constituents

Figure 1 reports the DSC records obtained from carob flour (CF) samples at different moisture content. The observed traces appear to be different depending on water availability. More specifically, we observe a trace involving only an endothermic contribution at the highest moisture percentage considered, which turns in a smaller endothermic peak followed by an increasingly more prominent exothermic event as the water amount is reduced.

Considering the composition of the carob flour here investigated, whose main constituents are proteins at about 23% and dietary fibres (mainly constituted by locust bean gum, LBG) at about 52%, we may argue that the endothermic effects might be ascribable to the protein fraction denaturation, whereas the

progressively more consistent exothermic events enhanced in water-limiting conditions are of difficult interpretation at this stage since many peculiar processes (protein aggregation, gelation, etc.) would be compatible with this picture (Fitzsimons et al., 2007; Goycoolea et al., 1995; Pelosi et al., 2019; Saitta et al., 2022).

In order to dissect the contributions to the complex behaviour reported in Figure 1, a deeper DSC investigation was carried out on the flour main constituents, *i.e.*, carob proteins (CP) and LBG, alone and in combination, as well as at various water content.

Figure 2a reports the DSC records obtained from carob protein (CP) samples at different moisture content. We observe endothermic profiles attributable to the thermal denaturation process undergone by the proteins, whose stability appears to be influenced by the percentage of water. Specifically, the lower the moisture content, the higher the temperature range of the thermal denaturation peak. Although the protein fraction is an ensemble of proteins, we assumed here for simplicity the observed T_{max} of the thermogram as an average index of protein stability as concerns the denaturation temperature in order to quantify the stability-versus-moisture trend. On the other hand, the denaturation enthalpy was almost unaffected by the different moisture percentages and is $\Delta_d H$ = (6.4 ± 0.6) J/g_{dry}. The T_{max} behaviour, *i.e.*, the entropic stabilization of proteins in water-limiting conditions, is in line with the literature and was also observed for pure protein systems (Saitta et al., 2020). We may note that no exothermic phenomena (typical index for protein aggregation) were observed even at the lowest moisture content.

Figure 2b reports the DSC records obtained from the analysis on LBG:CP blend (50:50 % w/w) at various moisture percentages. We mention here that any significant signal was obtained by DSC analysis performed on locust bean gum (LBG) samples alone (flat traces not reported) at both the moisture and the temperature ranges investigated. Indeed, despite gelling properties of LBG are often reported, LBG does not form gel on its own, but gels are formed with other hydrocolloids and/or gelling agents (kappa carrageenan, agar, etc.) (Goycoolea et al., 1995; Pegg, 2012).

For our blend, we observe a behaviour that somehow recalls the one shown by CF in Figure 1. Indeed, after the protein thermal denaturation, an exothermic phenomenon appears (except for the sample at the highest moisture considered) and becomes more relevant and closer to the protein denaturation temperature as the moisture decreases. Such relevant and sometimes superimposed exothermic events to the CP denaturation peak may influence the observed T_{max} of the thermogram that may be brought forward. However, for the sake of simplicity, we still consider this parameter as an average index of protein stability, being aware that the T_{max} values, in these cases, represent a lower limit.

Accordingly, as concerns the CP thermal stability, the protein fraction results to be again progressively stabilized as the moisture percentage decreases, but the trend of the denaturation peak maximum temperature, T_{max}, versus the water content (% w/w) seems to be different with respect to the CP system alone (Figure 3).

In particular, from the two trends shown in Figure 3, we observe that proteins in the blend system behave as they would experience an environment with less moisture than the nominal (Saitta et al., 2020). In other words, water is not homogeneously distributed among the protein and polysaccharide (LBG) phases, this last exhibiting a higher affinity for water than the protein fraction. Indeed, if we consider, for instance, the behaviour of the protein fraction in a 50:50 % w/w LBG:CP blend at 55% nominal sample moisture, we can observe from Figure 3 that the CP fraction's thermal stability is analogous to the one that would be exhibited by a CP sample alone at about 50% moisture. This means that, in term of water content, there is about a 45%/55% partition among the CP and LBG phases, respectively, in the considered LBG:CP blend at 55% nominal sample moisture, instead of a homogeneous 50%/50% water partition.

Instead, as regards the relevant post-denaturation exothermic events recorded for the 50:50 % w/w LBG:CP blends (Figure 2b), basing on the results shown in Figure 2a (absence of exothermic events), they seem not to be due to intrinsic aggregative properties of the protein fraction, since no aggregation is observed for CP even at very low water content. Accordingly, although a partial contribution due to protein aggregative phenomena cannot be totally excluded, we may argue that LBG chains might interact with the denatured protein population, and the interaction might induce the formation of a gel-like structure, which is commonly revealed as an exothermic peak on DSC thermograms (Fitzsimons et al., 2007; Goycoolea et al., 1995; Guigo et al., 2012).

In order to better highlight the interaction between CP and LBG fractions, Figure 4 reports the thermograms obtained for CP:LBG mixtures at different ratios selecting a severe water-limiting condition (about 30% moisture). We mainly observe a progressive lowering of the endothermic contributions due to the protein fraction denaturation as its percentage decreases, whereas the superimposed exothermic event becomes predominant as the LBG fraction increases.

To sum up the above results, we may say that the thermal transitions of the carob flour (CF) are mainly due to the protein thermal denaturation and to post-denaturation protein-polysaccharide interactions that produce an overall exothermic "gelation-like" event, whose extent severely depends on CP:LBG ratio. Furthermore, these transitions are moisture-dependent as concerns both the onset temperature and the extent of the exothermic phenomena.

As a consequence of such a complex thermal behaviour exhibited by CF, its inclusion as it is or as fractions in more complex food matrices might lead to technological aspects difficult to predict, often inducing to investigate products basing on the phenomenology and on a trial-and-error approach (Tsatsaragkou et al., 2014).

In this perspective, we tried to dissect the above-mentioned contributions by investigating the influence of both CF flour and CP and LBG fractions on the main macromolecules and phenomena involved in wheat flour during bread making, *i.e.*, on starch gelatinization and on gluten reticulation, in view of their potential use as ingredients for the production of carob-enriched bread.

3.2. Effects of carob ingredients on starch gelatinization in wheat flour

The DSC approach permitted an evaluation of starch gelatinization properties in the different systems and of their possible alteration upon the addition of carob ingredients (CF, CP, LBG) to wheat flour.

Although the percentage of carob ingredients would usually be small in real products such as bread, the mixtures analysed here were chosen with a high amount of carob ingredient, namely 50:50 % w/w wheat:carob-ingredient, to better emphasize the effects due to the mixing of the components. The systems selected were investigated at high moisture content and at limiting-water condition (70% and 40% moisture content, respectively) and the results are summarised in Figure 5.

Figure 5a reports the DSC thermograms obtained for the reference wheat system alone (W) and for blends prepared as 50:50 % w/w W:CF and W:LBG in a highly hydrated environment (MC of about 70%), *i.e.*, in conditions that prevent water competition effects.

As regards the reference system, we observe the typical main endothermic events corresponding to the different steps of starch gelatinization process. More specifically, the thermal profile involves a main peak that reflects almost the full starch gelatinization process, indicating that the immediately available water molecules of the sample are enough to gelatinize starch granules to completeness in just one step, whereas the second peak is mostly attributable to the amylose-lipid complexes dissociation (Emide et al., 2023). The overall enthalpy of gelatinization recorded is ΔH = (11.6 ± 0.6) J/g_{dry} and is within the typical enthalpy range shown for a common wheat flour (Fessas and Schiraldi, 2000).

The 50:50 % w/w W:CF blend at the same moisture content exhibits a similar thermal profile compared to the reference, with an unaffected gelatinization onset temperature and a main starch gelatinization peak followed by the amylose-lipid complex dissociation. However, considering the enthalpic contribution to the transitions, the main gelatinization peak enthalpy is coherent with the proportions of the constituents, whilst the second peak appears greater than the reference's, revealing that the highest temperature portion of this peak likely includes the contribution of the denaturation of the protein fraction constituting the CF. Indeed, the temperature range considered (around 105°C) is coherent with the T_{max} shown by CP in Figures 2a and 3.

Instead, the 50:50 % w/w W:LBG blend displays a thermal behaviour analogous to the reference one, with a an enthalpic contribution related to the W portion only, since no transition is observed for LBG. We may conclude that starch gelatinization is not affected by these carob ingredients when in a highly hydrated environment.

On the other hand, at water-limiting condition (MC of about 40%, Figure 5b), where the water competition becomes evident, the DSC thermogram obtained for W exhibits some differences if compared to the trace in Figure 5a. Indeed, the starch gelatinization process strongly depends on the overall moisture content, which is distributed among at least two phases, namely the starchy and the protein phases (gluten and

other wheat's globular proteins) (Caramanico et al., 2017; Fessas et al., 2008). Therefore, at these conditions, the mere gelatinization process results to be split into two steps, the first of which involves the immediately available water molecules of the sample, whereas the second step occurs at higher temperatures when further water molecules gain enough mobility to take part at the completion of the process (Fessas and Schiraldi, 2000). Clearly, the dissociation of amylose-lipid complexes still follows the second gelatinization peak and is upshifted towards higher temperatures. Moreover, the overall enthalpy of the process results greater than the one shown by the W sample at 70% of moisture content (ΔH = (17.0 ± 0.9) J/g_{dry}), in line with the literature (Fessas and Schiraldi, 2000). Accordingly, this thermogram represents the reference system to be compared with at 40% moisture conditions.

The 50:50 % w/w W:CF blend at MC of 40% shows a thermal profile different to the reference one. Firstly, we observe an exothermic event that occurs at high temperatures. According to the results of the previous section (3.1), we may argue that the exothermic event follows the protein denaturation, whose peak (at about 117°C) is less visible because of the superimposed amylose-lipid complex dissociation, and it likely reflects the interaction between CP and LBG fractions constituting CF, as already clearly indicated in Figures 1, 2b and 4. Secondly, we observe that the first gelatinization peak is much less pronounced (out of the constituent's proportion justification), whereas the second step is enhanced and shifted at higher temperatures. This is in an index of less hydrated starch granules, likely due again to the high water affinity shown by the LBG fraction constituting CF (Torres et al., 2012).

Indeed, similar effects are observed for the 50:50 % w/w W:LBG system. In this case, the first gelatinization peak is further reduced, whereas the second gelatinization and the remaining part of the process are further shifted at higher temperatures. By contrast, no exothermic event is observed, confirming that the protein fraction is involved in that mechanism.

In any case, the overall process enthalpy analysis indicates that the gelatinization eventually reaches the completion. In other word, the presence of the LGB severely influences the gelatinization process indirectly by only affecting the kinetics of the process because of an unfavoured water repartition, but no particular starch-LBG interaction is revealed. However, we remind here that the DSC results are performed with closed pans, *i.e.*, under constant moisture, whilst water evaporation continuously occurs in real bread-making, and a delayed starch gelatinization kinetics might have deleterious effects on the overall process.

Such a slowing down of the gelatinization kinetics depends on the amount of LBG and is detectable even in the presence of smaller LBG percentages (Figure S1 in the Supplementary Material).

As far as the 50:50 % w/w W:CP blend at 70% moisture is concerned (Figure 5c), both the starch gelatinization profiles and the gelatinization onset temperatures result to be still unaffected upon the addition of CP fraction if compared to the respective reference. Moreover, the CP denaturation peak is clearly detected and shows a denaturation temperature (T_{max}) of about 104°C, which is comparable to the CP denaturation temperature when alone at the same moisture condition (MC 70%, Figure 3). Both the

enthalpies of gelatinization and denaturation are coherent with the composition proportion. Both these enthalpic and the entropic aspects of CP denaturation peak reveal that the presence of starch and gluten does not influence the thermal stability of the carob proteins, which behave independently as far as no competition for water is present at such a high water content.

Moving at about 40% water content (Figure 5d), we observe that the CP denaturation peak is partially overlapped to the amylose-lipid dissociation phenomenon and the peak is positioned at a higher temperature (about 119°C), but again the upshift was of the same extent of that exhibited by CP alone at the same moisture (Figure 3). Furthermore, we observe a similar profile to the respective reference as regards the gelatinization process, and the gelatinization onset remains unaffected. Indeed, despite the first gelatinization peak seems slightly different (maybe due to the small amount of LBG present in this fraction), the second gelatinization peak and the amylose-lipid dissociation remain almost unaffected.

These data are in line with the general picture that emerges from the literature concerning the thermodynamic incompatibility between proteins and starch, according to which separated starch and protein phases that barely influence each other are present, apart for the competition for water that depends on the specific protein fraction (Caramanico et al., 2017; Tolstoguzov, 1997). Specifically, for the present carob protein fraction, we observe that no significant competition for water is established among these two main phases even in water-limiting condition.

To sum up, our data indicate that the carob flour, as it is, is of difficult use as ingredient for enriched wheat bread making for two reasons: the carob protein-LBG interaction that triggers the formation of a gel-like structure, and the inappropriate conditions for starch gelatinization due to the LBG water absorption.

On the other hand, the CP flour seems to be a suitable ingredient for enriched wheat bread (mostly for nutritional reasons) without severe contraindications about both the starch gelatinization process and the water partition between the starchy and protein phases, including gluten. Indeed, despite gluten transitions are not revealed by DSC, the above data represent an indirect indication that gluten hydration properties are unaffected as CP has been shown above to behave as it was alone.

Accordingly, since gluten reticulation process depends on hydration (Fessas and Schiraldi, 2001) (if equivalent mechanical stress is considered), we may argue, in first approximation, that the presence of CP does not significantly influence gluten reticulation as regards this aspect. Of course, in a real system, gluten hydration is only one of the many factors that influence the gluten reticulation process, and such an indication needs an experimental confirmation. Furthermore, the peculiar surfactant properties of the added protein may also play a significant role on the crumb alveoli structure of a CP-enriched bread (Fessas and Schiraldi, 1998). In other words, although the information obtained through the DSC investigation on CP-wheat model systems is usefull for the comprehension of the properties of bread prepared with such an ingredient, it is well known that the constituents behaviour and the concomitant effects may be more complex to predict when included in a real system with more complex matrix.

In order to asses the validity of the indication emerged from this study and the degree of resilience to side effects due to matrix complexity, we prepared and compared reference wheat-based loafs and ones enriched with 5% w/w of CP (Miñarro et al., 2012). The technological properties of these bread loafs are shown in Table 1 and in Figure S2 (Supplementary Material).

3.3. *Effects of carob proteins on wheat bread*

Bread slices obtained from both reference wheat bread and the one containing 5% w/w CP exhibit no significant differences if compared to each other in terms of loaf height and crumb alveoli distribution (Figure S2 in the Supplementary Material), confirming the above statement concerning gluten reticulation. Moreover, no volume impairment is observed (the residual LBG content present in carob germ flour is insignificant in these proportions, see section 2.1). The mere visive picture of this comparison about the possible impairment of gluten network is also confirmed by data reported in Table 1 concerning the crumb texture and structure, more specifically in terms of crumb firmness and relative elasticity as well as of porosity.

In particular, we observe that CP addition does not influence neither the crumb firmness nor the consistency; analogously, crumb's structural properties resulted not to be significantly affected by the presence of carob proteins and both the number and the alveoli dimension remain the same. These data indicate that the surfactant effect of the proteins is small to be evident and that the gluten network is not affected by the presence of CP fraction at the percentage proposed.

As regards the moisture content of breads, Table 1 shows the mean values obtained for the bread crumb and crust. The moisture content of the wheat bread crumb and crust with 5% CP do not present statistically significant differences with control breads. This is in accordance to the findings of the previous DSC thermographs, where it is evident the thermodynamic incompatibility of CP with starch and the absence of water redistribution among phases.

Despite we may not exclude some counterbalance effects, the general picture emerged from this real system validation is in line with the suggestions emerged from the calorimetric investigation.

Nevertheless, this study was performed using materials from a single carob source. Considering that the properties of both the main ingredients of this system (CP and wheat flour) may depend on the genotype and plant growth conditions of the seed used for the production of the flours (Emide et al., 2023), the peculiarities of the properties here assessed might slightly vary, though, to the best of our knowledge, no thermodynamic evidences are reported in the literature about the effects of different carob genotypes on analogous wheat/carob blends for a proper comparison. In any case, the overall scenario and the conclusions based on the calorimetric results are expected to have a good degree of generality since the thermodynamic incompatibility between such a type of biopolymers is well consolidated (Fessas et al., 2008; Tolstoguzov, 1997).

Deeper technological characterizations and formulation aspects are beyond the scope of this paper that is mainly devoted to assess and dissect the interplay between carob and wheat flour macromolecules following their thermal transitions in complex matrices.

4. Conclusions

In this work, we firstly characterized the thermal properties of carob flour (CF) and specific derived ingredients, namely carob protein fraction (CP) and locust bean gum (LBG), through DSC as a function of the water content. The results indicated that the thermal transitions of the carob flour (CF) are mainly due to the protein thermal denaturation and to post-denaturation protein-LBG interactions that produce an exothermic "gelation-like" event depending on CP:LBG ratio. Water limitation pushes the onset of protein denaturation at higher temperatures and enhances the overall exothermic effect.

Such an initial analysis permitted to dissect the influence of carob ingredients on the properties of wheat-based systems, with the purpose of considering their introduction into enriched baking products. The results highlighted that the presence of carob flour alters the water distribution in wheat-based blends, due the high affinity of LBG for water, and negatively affects the starch gelatinization kinetics. Furthermore, the concomitant presence of LBG and carob proteins still triggers the formation of a gel-like structure. Both these effects indicate that the carob flour, as it is, is of difficult use as ingredient for enriched wheat bread making.

On the other hand, the DSC results indicated that the presence of CP does not significantly influence the water distribution between the starchy and protein phases (including gluten) in the wheat-blend system and suggests that the carob protein presence does not compromise the technological performance of the enriched dough. This prediction was tested through technological trials by preparing and comparing reference wheat loafs and ones enriched with 5% w/w of CP. The analysis of such trials concludes that the enriched bread remains comparable to the reference's, confirming the predictions emerged from the DSC study.

5. Acknowledgements

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Tables

Table 1: Technological properties of wheat bread (reference) and carob protein-enriched wheat bread (containing 5% w/w CP).

		Reference	*+ 5% w/w Carob proteins*
Water content	CRUST (g H2O/100g crust)	19.82 ± 2.69	19.15 ± 0.97
	CRUMB (g H2O/100g crumb)	39.44 ± 3.66	41.02 ± 3.61
Crumb firmness	COMPRESSION-MAX LOAD (N)	15.09 ± 5.56	12.71 ± 1.03
	RELATIVE ELASTICITY(%)	46.45 ± 5.96	43.58 ± 1.22
Crumb structural characteristics	POROSITY(%)	71.80 ± 4.18	70.67 ± 3.61
	SURFACE POROSITY(%)	32.63 ± 5.00	32.07 ± 3.20
	Average Cell Diameter (mm)	0.377 ± 0.025	0.374 ± 0.025
	N° of Cells/cm	89 ± 10	91 ± 8

Figure captions

Figure 1: DSC traces obtained for carob flour at different moisture contents.

Figure 2: DSC traces obtained for a) carob protein (CP) and b) locust bean gum : carob protein blend (LBG:CP 50:50 % w/w) at different moisture contents (the purple trace is shown as full scale in Figure 4). LBG samples did not produce any DSC signal at the same conditions applied for both CP and LBG:CP blend. Dashed grey lines are included in the figure to facilitate the comparison of the protein denaturation peaks between panels a and b.

Figure 3: The maximum temperature (T_{max}) of the DSC protein denaturation peaks vs water content (% w/w) for carob protein (CP, in blue) and locust bean gum : carob protein blend (LBG:CP 50:50, in red). Black arrows are also shown to better clarify the water redistribution in the mixture considered.

Figure 4: DSC traces obtained for doughs prepared as LBG:CP mixtures at different ratios and at about 30% moisture content. The DSC trace obtained for CP alone at 30% moisture (black dashed trace) is reported again from Figure 2a for the sake of comparison.

Figure 5: DSC traces obtained for wheat (W) doughs alone (blue curves) and in the presence of 50% w/w of carob flour (CF, green curves), carob proteins (CP, red curves) and locust bean gum (LBG, purple curves) at different moisture content (MC of about 70% in panels a-c and 40% in panels b-d). Dashed grey lines are included in the figure to facilitate the comparison of the gelatinization and the protein denaturation peaks between panels.

Figures

Figure 1

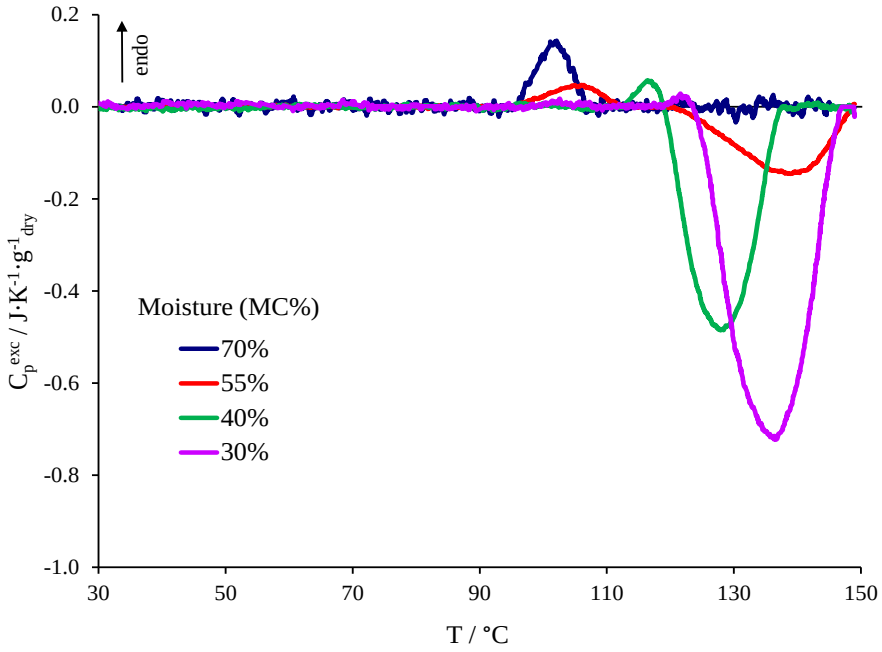


Figure 2

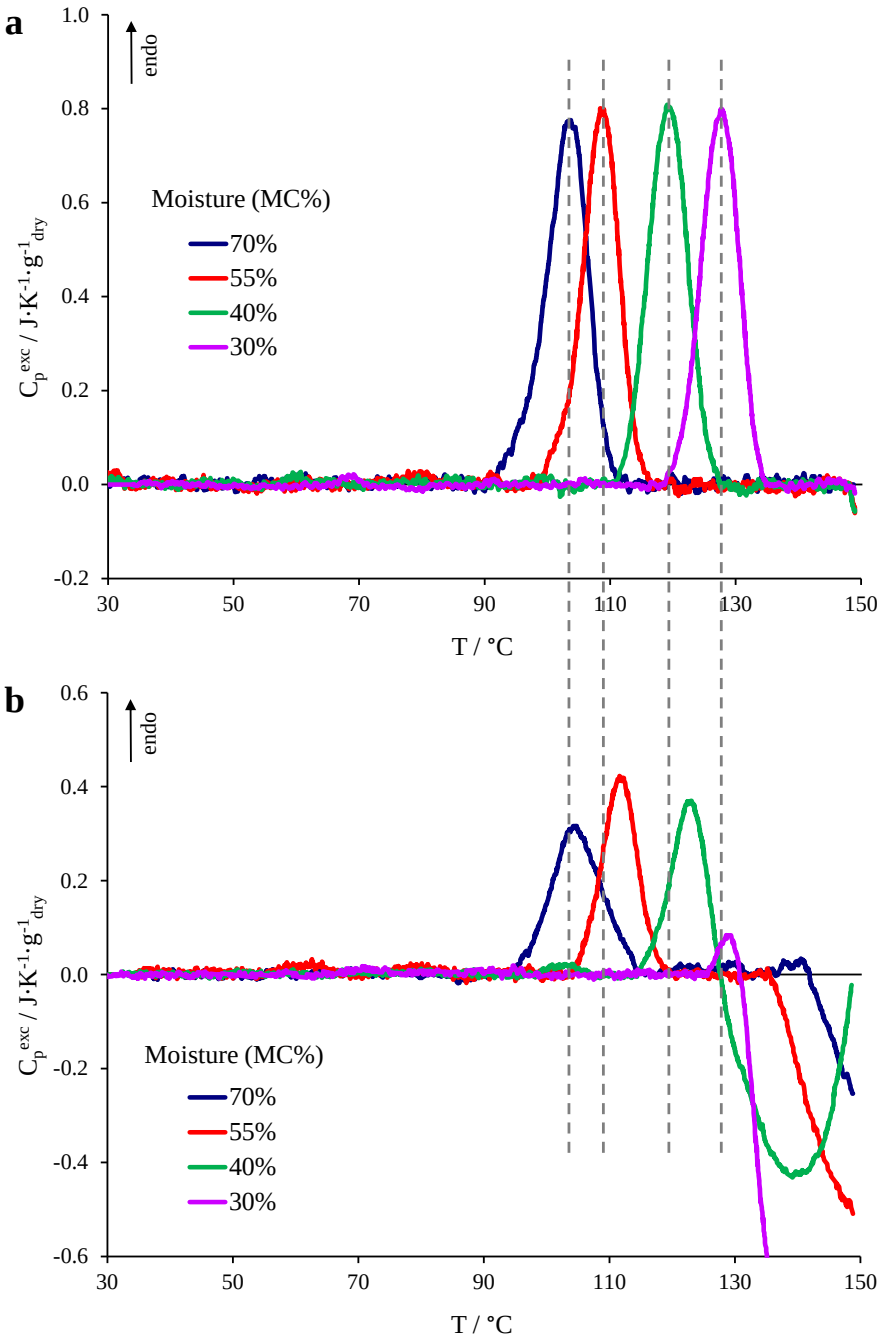

a
endo
Moisture (MC%)
70%
55%
40%
30%
C_p^{exc} / J·K^{-1}·g$^{-1}_{dry}$
T / °C
b
endo
Moisture (MC%)
70%
55%
40%
30%
C_p^{exc} / J·K^{-1}·g$^{-1}_{dry}$
T / °C

Figure 3

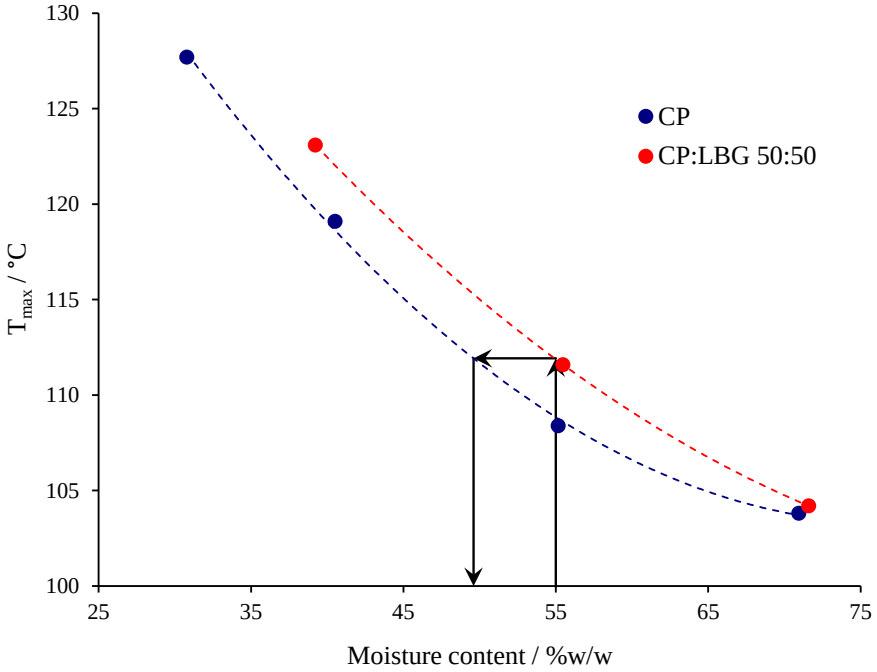
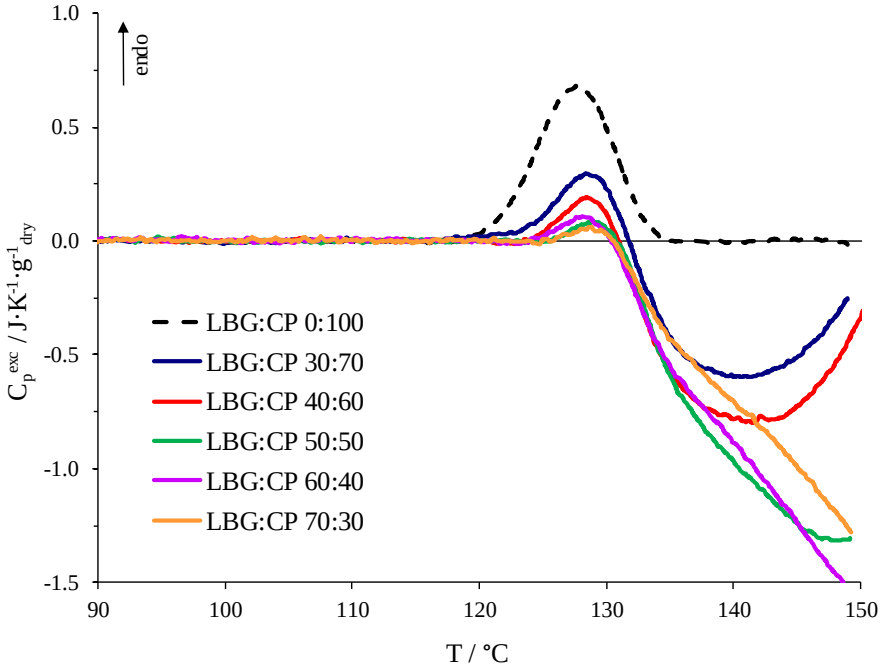
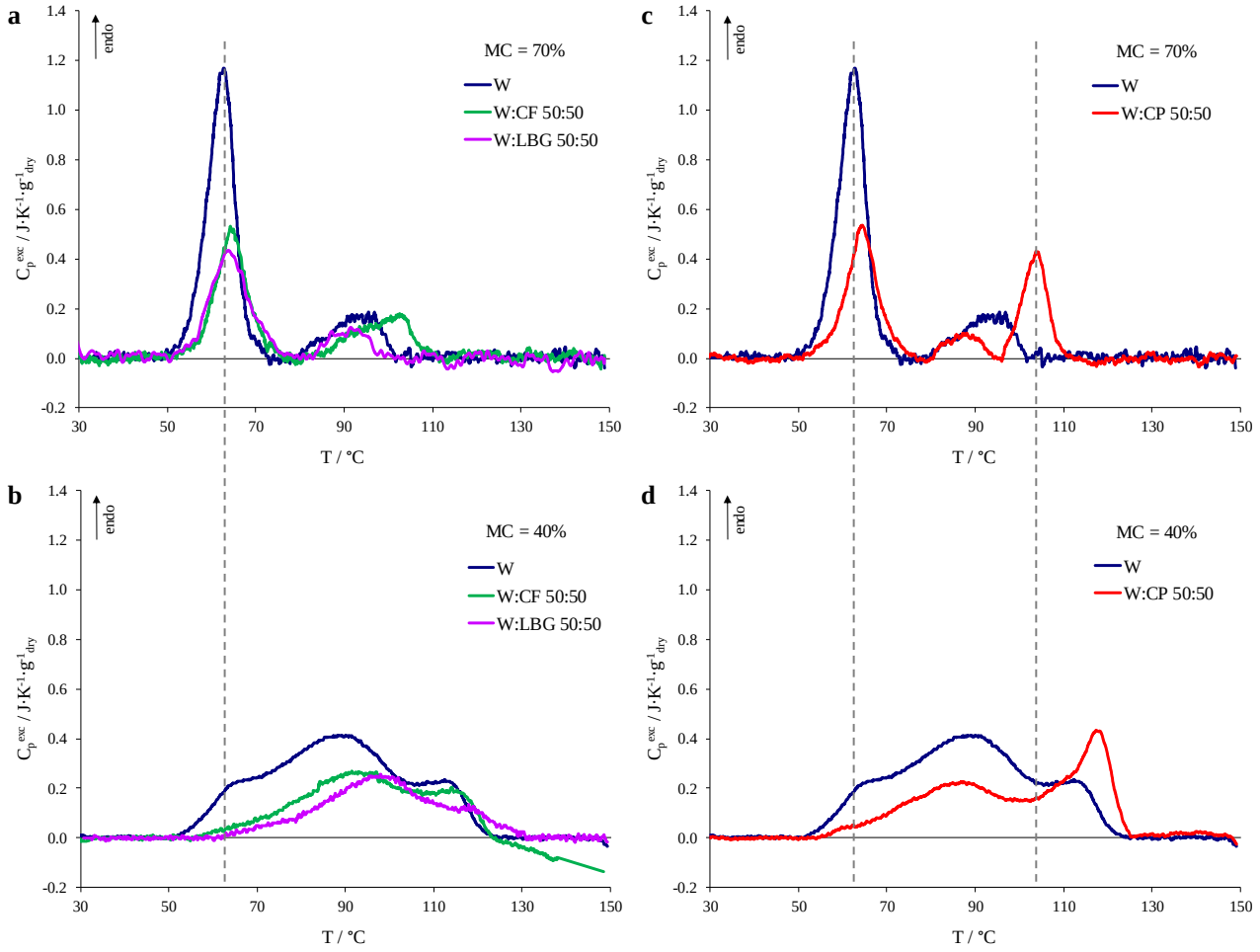


Figure 4



1 **Figure 5**


a
endo
MC = 70%
W
W:CF 50:50
W:LBG 50:50
C_p^{exc} / J·K^{-1}·g$^{-1}_{dry}$
T / °C
c
endo
MC = 70%
W
W:CP 50:50
C_p^{exc} / J·K^{-1}·g$^{-1}_{dry}$
T / °C
b
endo
MC = 40%
W
W:CF 50:50
W:LBG 50:50
C_p^{exc} / J·K^{-1}·g$^{-1}_{dry}$
T / °C
d
endo
MC = 40%
W
W:CP 50:50
C_p^{exc} / J·K^{-1}·g$^{-1}_{dry}$
T / °C

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3