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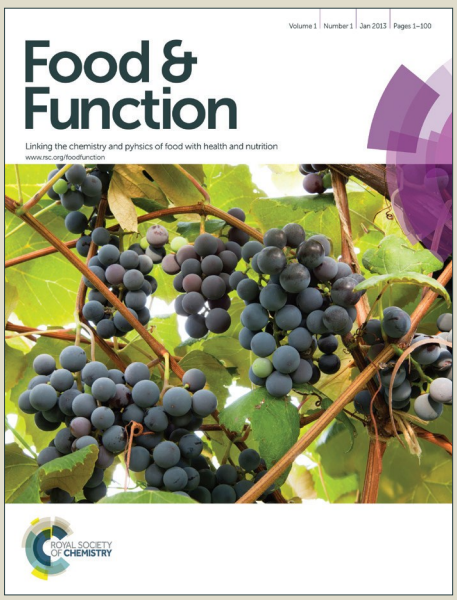

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The impact of raw materials and baking conditions on Maillard reaction products, thiamine, folate, phytic acid and minerals in white bread

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Abstract

The aim of this study was to develop a white bread with improved nutrient contents and reduced levels of potentially harmful Maillard reaction products such as N^{ε}-carboxymethyllysine (CML) and 5-hydroxymethylfurfural (HMF). Essays were carried out through a full factorial experimental design allowing the simultaneous analysis of four factors at two levels: 1- wheat flour extraction rates (ash contents: 0.60% - 0.72%), 2- leavening agents (bakers' yeast - bakers' yeast and sourdough), 3- prebaking and 4- baking conditions (different sets of time and temperature). The baking conditions affected HMF and CML as well as certain minerals contents. A reduced baking temperature along with a prolonged heat treatment was found to be favourable for reducing both CML (up to 20%) and HMF concentrations (up to 96%). The presence of sourdough decreased the formation of CML (up to 28%), and increased the apparent amounts of calcium (up to 8%) and manganese (up to 17.5%) probably through the acidification of the dough. The extraction rate of flours as well as interactions between multiple factors also affected certain minerals amounts. However, compounds like folate, thiamine, copper, zinc, iron and phytic acid were not affected by any of the factors studied.

Introduction

Modifications in eating habits combined with the rise of new bread-substitutes have caused a decrease in bread consumption in recent years[1]. In order to counter this development, dietary guidelines insist on the need to increase the intake of cereal foods such as bread in diets[2]. Bread has generally a low lipid content and is rich in nutrients such as dietary fibres, vitamins, phytochemicals and minerals[3]. The synergic activity played by bread nutrients has been associated with decreasing risks of several chronic diseases such as diabetes and cardiovascular diseases[4].

Conversely, the baking process leads to a partial deterioration in the nutritional quality of the cereals. According to Bourre et al[3], breads contain 10 to 50% fewer B vitamins than the original flours. Thiamine (vitamin B1) and folate (vitamin B9) are water soluble vitamins whose roles in physiological processes are fundamental. Thiamine contributes to the carbohydrate metabolism as a cofactor for several enzymes while folate contributes to amino acid and nucleic acid biosynthesis. Humans are auxotrophic for these vitamins and have to draw them from foods, in particular cereals and yeast[5,6]. However these vitamins are partly lost during the refining and processing of foods. Minimizing these losses is thus a critical point for food industrials.

Baking also generates several unwanted Maillard reaction products (MRPs)[7]. The latter include acrylamide which is classified as a probable carcinogenic[8], and 5-hydroxymethylfurfural (HMF) which is suspected of being a potential cancer promoter[9]. N^{ε}-carboxymethyllysine (CML), another MRP encountered in bread, is equally associated with multiple pathological conditions[10,11] and is often used as a marker for advanced MRP formation in foods[12].

The health impacts of dietary MRPs are currently being debated. Studies on complex heated foods do not allow definitive conclusions to be drawn about the impact of MRPs on health[13], and toxicological data on specific MRPs vary widely so that the risk level associated with their dietary intake is unclear[14]. Until proper conclusions can be reached about the health impact of some MRPs, the ALARA principle (as low as reasonably achievable) should be applied to mitigate their formation in bread as much as possible.

Another undesirable compound present in cereals and thus in bread is phytic acid. Certain favourable health impacts have been attributed to phytic acid[15] but it is still considered as an anti-nutritional compound. It binds and chelates minerals into insoluble complexes resulting in a decrease of minerals bioavailability and absorption. Phytic acid can also form complexes with proteins which reduces their activity and digestibility.

In order to regain part of the consumers' interest, new breads are being developed with various health and nutritional attributes[16]. The aim of the current study is to develop a white bread with improved nutrient contents and reduced levels of potentially harmful MRPs. Essays were carried through an experimental design allowing the simultaneous analysis of four factors thought to affect the final quality of bread: the wheat flour extraction rate, the leavening agent, the prebaking and baking conditions.

The extraction rate of flours is determined by ash content. It is a function of the milling procedure and it is known to impact the organoleptic characteristics and nutritional profile of the final product. During the second half of the twentieth century consumers and bakers were oriented towards white breads, favouring the flours generating the whitest crumb and requiring the fewest bleaching agents[7,17]. However white breads tend to loose most of the nutritional value of the wheat grains[17]. A great deal of work has been published in recent years dealing with whole grain breads[19,20,21]. However, white breads remain a major staple food favoured by many consumers[22]. By comparing two extraction rates used for white breads in France, flour types T55 and T65, combined with other bread-making factors, our aim was to determine the ideal combination for a nutritionally superior white bread with no additive.

Fermentation is also a crucial step in bread-making carried out by either baker's yeast, *Saccharomyces cerevisiae*, or by a combination of microorganisms present in sourdoughs, particularly lactic acid bacteria. It ensures the rise of the dough and plays a role in the flavour profile of the bread[23]. The leavening agents have differing impacts on the final quality of the breads, both on a sensory level and on a functional level. Sourdoughs for instance have been reported to delay bread firmness and staling[24] and to decrease phytic acid contents while improving magnesium and phosphorus solubility in breads through acidification of the dough[25,26]. This acidification can also be expected to have an impact on the

93 formation of certain MRPs[27]. However, sourdough poses certain technical difficulties concerning
94 activation and fermentation times so it is important to compare both leavening agents and their
95 interactions with other factors on the quality of the finished bread.
96 The need to purchase bread on a frequent basis due to its poor preservation aptitudes has negative
97 effects on bread consumption. A palliative solution to these constraints is prebaked breads. Such
98 breads can be stored for longer periods than regular breads but they require a final step of baking.
99 Prebaking and baking help set the product's structure, physical characteristics and flavour profile[7].
100 During these two unitary operations the temperature of the crumb rarely exceeds 100°C while the
101 temperature of the crust may reach temperatures slightly lower than that of the oven. Thus, the
102 caramelization and the Maillard reaction occur mainly in the crust of breads generating most of the
103 breads' characteristic aroma. However, while aromas are one of the beneficial side effects of the
104 Maillard reaction, other more controversial MRPs can also be generated. Finding the adequate
105 combination of time and temperature for both operations is one of the mitigation strategies for
106 unwanted MRP reduction in breads.
107 To our knowledge no study to date has addressed the impacts of raw materials and baking conditions
108 of bread on vitamins, minerals and MRPs amounts simultaneously.
109

Materials & methods

Reagents

Reagents were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and Sodipro (Echirolles, France) unless otherwise mentioned. All reagents used were of analytical grade.

Design of experiments and statistical analysis

The experimental design was defined as a result of an initial screening design (Plackett–Burman design not shown) to determine the most influential factors on the vitamins, minerals and MRPs that were measured (data not shown). Factors such as extraction rates of flours, leavening agents, fermentation conditions, kneading conditions, salt amounts, enzyme mixes as well as prebaking and baking conditions were tested. Only four out of eight factors had a significant impact on the responses measured and thus were reassigned in a 2^4 full factorial design (Table 1). These four factors and their levels are: the extraction rate: -1=T55 & 1=T65; the leaving agent: -1=yeast & 1=yeast and sourdough; the pre-baking conditions: -1=4min at 250°C & 1=13min at 130°C; and the baking conditions: -1=12min at 200°C & 1=9min at 250°C.

Four repetitions were made at a pseudo central point (due to non-quantitative factors) in order to estimate experimental standard deviation. These repetitions were made using a T65 flour and a combination of yeast and sourdough as leavening agents. They were prebaked at 250°C for 4 min and the final baking was made at 250°C for 9 min. The bread preparations were carried out in the randomized order set in Table 1 and one repetition was made for each of the sixteen combinations. Data were collected and analysed using Microsoft Excel (2010) by a multiple regression model including mean effect for each factor and all possible interactions between, two, three and four factors. Significant effects were detected using confidence interval method, at 95% confidence level. If the zero value was included into the interval, the corresponding effect was declared non-significant. In the

following text, a significant mean effect for a factor was described by the ratio between (mean value at +1 level minus mean value at -1 level) divided by the overall mean of the response studied. This ratio was expressed in percentage and indicated how high was the variation of the response, due to variation of the factor. A significant interaction between two factors indicated that the variation of the response due to variation of one factor was different according to the level of the second factor. The same was true when the effect of the combination of two factors was studied according to the levels of a third factor.

The target concentration for each compound (the response) was selected according to its detrimental or nutritional interest. For the compounds that should be as low as reasonably achievable (CML, HMF and phytic acid) the target value was 0. For the nutritionally desirable compounds (minerals and vitamins), the objective was to increase their values, thus the target value was the maximal value achievable. After normalization of all data in the range of [0,1], the target/optimal bread should have CML, HMF and phytic acid contents at 0 and minerals and vitamins contents at 1 (Fig.1, green line).

Bread-making

Breads were prepared at a pilot scale using the experimental design shown in Table 1. Two hundred breads were prepared per condition. Bread dough was prepared using wheat flours at two extraction rates used for white breads in France, flour types T55 (ash content 0.60%) and T65 (ash content 0.72%). The leavening agent used was either yeast alone (1.5%) (Lesaffre, Marcq-en-Baroeul, France), or yeast combined with a sourdough obtained with wheat middlings (5%) (Philibert Savours, Crottet, France). The salt level was set to 1.3% and the hydration level was 57 and 54%, depending on the flour extraction rate. The ingredients were mixed for 4 min at low speed then for 4 min at high speed in a spiral kneader (Diosna, Osnabrück, Germany). Dough portions were bulk fermented at ambient temperature for 2 h and then proofed for 1.5 h at 28°C and 80% humidity. Breads were prebaked in a ventilated oven (Pavailler, Portes-lès-Valence, France) according to the experimental design (13 min at 130°C or 4 min at 250°C). The lower temperature was chosen as the lowest

160 temperature allowing the necessary phenomena involved in the formation of the crumb (evaporation of
161 water, coagulation of proteins, gelling of starch, inactivation of yeast and enzymes…). The highest
162 temperature was the one used routinely by the industrial bakery. The obtained prebaked breads were
163 then frozen (45 min at -35°C in a Panem deep freezer, La Crèche, France) before being baked using a
164 ventilated oven (Pavailler, Portes-lès-Valence, France) according to the experimental design (12 min
165 at 200°C or 9 min at 250°C). The two baking conditions selected were set to be close to the real
166 conditions for the industrial bakery and to obtain a satisfactory level of browning on the crust. Final
167 breads weighed 64.95±1.88 g.

168

169 ## Crust/whole bread ratio and dry matter

170 Ten breads were selected randomly per condition and weighed. Crusts were separated from the crumb
171 and the collected crusts were weighed. A crust/whole bread ratio was calculated.
172 Dry matter levels (DM) were obtained by gravimetric determination (AOAC, 1990, method 925.10) in
173 quintuplicate.

174

175 ## Colorimetric analysis

176 Colorimetric measurements were achieved in triplicates on crust using a portable colorimeter CR-400
177 (Konica Minolta, Japan) previously calibrated on a calibration plate with the D65 light standard.
178 During analysis, all natural light sources were killed in order to improve the repeatability and the
179 reliability of measurements. Results were expressed in the international colour space CIE L^* a^* b^*,
180 where L^* stands for Lightness between 0 (black) and 100 (white). The coordinates a* and b* are not
181 presented in this study due to their lack of correlation to the analysed factors.

182

Protein analysis

Total protein amounts were determined by nitrogen conversion. Samples (80 mg) were analysed according to the Dumas method using a FP528 nitrogen analyser (LECO, Garges les Gonesse, France). Analyses were carried in triplicate according to the AOAC method 990.03. A conversion factor of 5.7 was used for the calculation of the protein content expressed in g/100 g of DM. Protein analyses were carried out on crust, crumb and the whole bread. The results were identical when expressed in g/100g of DM (data not shown).

Lysine and CML analysis

Lysine and CML analyses were done according to Niquet-Léridon and Tessier[28]. In brief ground bread amounts equivalent to 10 mg of proteins were reduced in triplicate with 1.5 mL of borate buffer (200 mM, pH 9.5) and 1 mL of sodium borohydride (1 M in NaOH 0.1 M) at room temperature for 4 h. Afterwards, protein hydrolysis was carried by adding 6 M HCl and incubating at 110°C for 20 h. Hydrolysates were vacuum-dried and dry residues were reconstituted with 20 mM nonafluoropentanoic acid containing adequate amounts of the internal standards (D_2)-CML (PolyPeptide Laboratories France SAS, Strasbourg, France) and ($^{15}N_2$)-lysine (CortecNet, Voisins-le-Bretonneux, France). Analyses were performed using a Surveyor HPLC system coupled by an electrospray ionisation (ESI) interface, to a Finnigan LTQ ion trap mass spectrometer working in its tandem operation mode (ThermoFisher Scientific, Villebon sur Yvette, France). The separation was made using a Hypercarb column (100 × 2.1 mm, 5 µm) and a 0.2 mL/min gradient of 20 mM nonafluoropentanoic acid in water and acetonitrile. The ESI interface operated in positive mode and tandem MS analyses were carried out in multiple reactions monitoring mode. Lysine and CML amounts were determined through peak ratios to their corresponding internal standard and standard curves comparison. The CML range used was 0-0.2 µg/mL, for a peak ratio varying between 0 and 2. The lysine range used was 0-0.75 µg/mL for a peak ratio varying between 0 and 3.

Free HMF analysis

HMF analyses were carried out in triplicate according to the method reported by Garcia-Villanova et al.[29]. Ground bread crusts (400 mg) were suspended in 7 mL of ultra-high quality water, vortexed for 1 min and centrifuged for 10 min at 5000 g. The extraction procedure was repeated three times and the three supernatants were grouped and purified with 0.5 mL of each Carrez reagent (I and II). After a final centrifugation at 5000 g for 10 min, the supernatants were completed to a final volume of 25 mL with ultra-high quality water. The solution was then filtered through a 0.45 µm nylon membrane filter and injected into a Surveyor HPLC system coupled to a Surveyor PDA detector (ThermoFisher Scientific, Villebon sur Yvette, France). The separation was carried out with water and acetonitrile (95:5; v/v) at a 1 mL/min isocratic elution flow using a Luna C18 column (250 × 4.6 mm, 4 µm) (Phenomenex, Le Pecq, France). The detection was made at 284 nm. The external standard method was used to determine the concentration of HMF in bread crusts. The responses of calibration standards (1–20 µg/mL) were used to draw the calibration curve and to evaluate its linearity. In order to avoid dilution of HMF during sample preparation only crusts were used for the quantification. The results were then expressed in whole breads using the crust/whole bread ratio, after verifying the absence of HMF in the crumb.

Thiamine analysis

Thiamine was analysed in breads according to a modified version of the method reported by Batifoulier et al.[5]. In brief, 2.5 g of breads was suspended in 25 mL HCl (0.1 M) in triplicate and dispersed using a disintegrator (Polytron) before being incubated at 100°C for 1h. After cooling, the pH was adjusted to 4 using NaOH (0.2 M) and 100 mg of takadiastase was added per gram of sample. The enzymatic hydrolysis was performed for 17 h at 37°C. The samples were then centrifuged at 9000 rpm for 10 min. Supernatants were collected and filtered through a 0.45 µm nylon membrane filter and 0.5 mL was mixed with an equivalent volume of potassium hexacyanoferrate. A SepPack C18 cartridge (Waters Corporation, Milford, MA, USA) conditioned with 5mL of distilled water and 2mL of methanol was used to purify the samples. The cartridge was then washed with 1 mL of sodium

acetate (0.05 M) and eluted with 0.5 mL of methanol/distilled water (70/30, v/v). The eluate was analysed using an HP 1090 serie II HPLC system coupled to an HP 1046 A fluorescence detector using a Waters Symmetry C18 column (150 mm x 4.6 mm, 5µ). The elution was made with methanol/phosphate buffer (pH 3.5) (70/30, v/v) at a flow rate of 1 mL/min. Thiamine was determined as thiochrome using fluorescence measurements at the excitation-emission wavelengths of 366-435nm. The concentration of thiochrome was determined using an external standard calibration curve (0.05 to 0.5 µg/mL).

Folate analysis

Folate was analysed in breads according to the NF EN 14131 norm (AFNOR, 2004) for determining folates in foodstuff using a microbiological assay. In brief, folic acid was extracted from breads using an overnight enzymatic hydrolysis at 37°C and pH 6.1 using gamma-glutamyl hydrolase. The enzyme was deactivated by heating at 100°C and samples were filtered and added to folate-free culture medium tubes, the Folic Acid Assay Medium (Becton, Dickinson and Company, Le Pont de Claix, France). After sterilization, each tube was inoculated with *Lactobacillus casei, subp. Rhamnosus* (ATCC 7469) and incubated at 37°C for 18 h. Bacterial growth was determined by turbidimetry and compared to the growth obtained for different concentrations of the standard solution incubated simultaneously and under the same conditions.

Phytic acid analysis

Phytic acid (phytate; myo-inositol 1,2,3,4,5,6, hexakisphosphate) was measured as phosphorus released by enzymatic activity using the Megazyme test-kit K-PHYT 05/07 according to the manufacturer's recommendations (Megazyme, Wicklow, Ireland). In brief, 1 g of bread was suspended in 20 mL HCl (0.66 M) in duplicate and mixed vigorously overnight at room temperature for acid extraction. The suspension was then centrifuged at 13000 rpm for 10 min and 0.5 mL of the supernatant was neutralized with an equivalent volume of NaOH (0.75 M). The enzymatic

dephosphorylation was then carried on through two stages: first by adding sodium acetate buffer (0.2 M, pH 5.5) and the phytase and incubating at 40°C for 10 min, then by adding glycine buffer (0.4 M pH 10.4) and the alkaline phosphatase in presence of $MgCl_2$ (4 mM) and $ZnSO_4$ (0.4 mM) and incubating at 40°C for 15 min. Trichloroacetic acid (50% w/v) was then added in an equivalent volume to the sample and the preparation was centrifuged at 13000 rpm for 10 min. The supernatant was used for colorimetric determination by measuring the absorbance at 655 nm and an external standard calibration method was used for the quantification (range 0 -7.5 µg of phosphorus).

Minerals analyses

Minerals analyses were carried out by an accredited analytical laboratory (USRAVE unit, INRA, Villenave d'Ornon, France) according to Cyprien et al.[30]. Briefly, bread samples were dry ashed and solubilized using nitric acid. Samples were then analysed using an ICP-AES 725 ES (Varian, Mulgrave, Victoria, Australia) equipped with a V-Groove nebulizer. Ca, Cu, Fe, K, Mg, Mn, P & Zn were analysed at the following wavelengths 422.6, 327.4, 259.2, 766.5, 285.2, 259.3, 177.4 and 213.9 nm respectively. Blanks using an in-house laboratory reference sample V463 (entire plant of maize) and having undergone the entire process from dry ashing to instrumental analysis were used as controls. Values obtained for these blanks were subtracted from concentration values measured in unknown samples. An external standard calibration curve was used for quantification of each mineral.

Results & Discussion

Table 2 shows certain characteristics of the 16 breads such as the content of the dry matter and those of crude protein and lysine as well as the crust/whole bread ratio. These values were further used for the expression of MRPs in various units such as the amount of CML relative to the protein or the lysine contents, and the amount of HMF (measured only in the crust) relative to the whole bread.

286 It is to be noted that acrylamide amounts were measured in the design breads and were below the
287 detection limit (18 ppb) of the current method used for acrylamide analysis[31]. It is therefore safe to
288 assume that, under the current conditions of formulation and bread-making of the design, acrylamide
289 amounts are too low to be a health or industrial issue.

290

291 ## Carboxymethyllysine

292 In most food matrices CML formation appears to be highly dependent on the amount of simple sugars
293 and available lysine which are the major two precursors of this MRP[28]. Both compounds are known to
294 be present in limited concentrations in wheat products[7]. As can be seen in Table 3, CML amounts
295 ranged between 0.70 and 0.97 mg per 100 g DM corresponding to 0.41 and 0.66 mg per 100 g of fresh
296 bread. If compared to the database from Hull et al.[32] the amounts of CML in the breads of the
297 experimental design are similar to the average presented for white breads (0.66 mg/100g bread).
298 However, when data are expressed using different units, the current breads have a significantly lower
299 amount of CML per kg of proteins and per mole of remaining lysine compared to Hull et al.'s study.
300 Such differences may be explained by the higher protein content and subsequently higher lysine
301 content of the raw materials used in the current study.
302 Taking into consideration a daily CML intake between 5 and 10 mg/day/person[33] and a bread intake of
303 approximately 170 g/day/person in Europe[34], the breads tested would be contributing from 7 to 22 %
304 of the daily CML intake. The ICARE clinical study on healthy adults also reported that bread
305 contributed to 27% of the food exposure to CML[33]. Even though CML levels in bread are not as
306 elevated as in other food matrices[32], the relatively high daily intake of bread in most countries makes it
307 a significant source of CML in the diet.
308 The analysis of the experimental design showed that the extraction rate of the flour did not affect the
309 final amount of CML. It can be explained by the fact that the two flours compared in this study did not
310 show any significant difference in the protein and lysine contents (Table 2). The T55 flour contains
311 12.68±0.6 g protein/100g DM and 0.40±0.02 g lysine/100g DM while the T65 flour contains
312 12.44±0.44 g protein/100g DM and 0.42±0.02 g lysine/100g DM.

313 While the prebaking step did not significantly affect CML content, the final baking step is of great
314 importance in its formation in breads. The breads baked at 250°C for 9 min (mean value: 0.87 mg
315 CML/100g DM) have systematically higher CML amounts than the ones baked at 200°C for 12 min
316 (mean value: 0.72 mg CML/100g DM).The mean significant effect corresponded to -19.6% of the
317 overall mean value. A similar observation was made in the study by Claus et al.[35] where reduced
318 baking temperature and prolonged heat treatment were found to be favourable for reducing acrylamide
319 levels. The importance of the baking step on final CML amounts was predictable since the pathways
320 leading to the generation of CML are known to be heat dependent. It should be noted that breads were
321 analysed only at the final point and that the impact of the pre-baking on the CML amounts may have
322 been masked by the final baking step. The multitude of possible pathways for CML formation, from
323 low to high temperatures, render very difficult to exclude the potential impact of the pre-baking step
324 on CML amounts. Further analyses are required before such conclusions can be drawn.
325 Another factor that influences the formation of CML is the leavening agent. Yeast leavened breads
326 have higher CML amounts than breads made using a combination of yeast and sourdough. The breads
327 made with yeast alone have higher CML amounts (mean value 0.84 mg CML/100g DM) than the
328 breads made with the combination of yeast and sourdough (mean value 0.75 mg CML/100g DM). The
329 mean significant effect represented in this case -11.3% of the global mean.
330 No significant interaction between factors was observed. However Tables 1 & 3 show that the bread
331 with the highest CML content was made using yeast as a leavening agent and baked at 250°C for 9
332 min (B02: 0.97 mg CML/100g DM) while the bread with the lowest CML content was the bread made
333 with yeast and sourdough and baked at 200°C for 12 min (B04: 0.70 mg CML/100g DM).
334 There are several explanations for the role played by sourdough on CML content. The most likely is
335 that the acidification of the dough, when using sourdough, may have slowed or modified the Maillard
336 reaction[27] and therefore generated lower amounts of CML. Another explanation is that the
337 microorganisms of the sourdough selected and metabolized different amino acids compared to yeast[36].
338 The absolute amounts of lysine might be reduced, producing lower levels of CML. Another hypothesis
339 is that reactive free amino acids might have been released from protein hydrolysis thus competing with
340 lysine and causing lower amounts of CML while generating other MRPs not measured in this study.

As no interaction between factors was detected, the results indicate that using sourdough as a leavening agent and a combination of low temperature - longer baking duration are the ideal strategies for mitigating the CML content in breads.

5-hydroxymethylfurfural

HMF amounts, shown in Table 3, varied between 0.02 and 0.94 mg/100g DM (0.016-0.67 mg/100g fresh bread). No traces of HMF were detected in the crumb of the breads studied (below the limit of detection which is 0.06 mg/100g). These amounts are lower than those reported by Ramírez-Jiménez et al.[37] for HMF contents in white breads (0.34-6.88 mg HMF/100g DM). These authors also showed that the amounts of HMF in the crust of breads can be 23.7 to 103.5-fold the amount measured in the crumb of the same bread. In our study, since the amounts of HMF in crusts are already low, it can explain the lack of detectable HMF in the crumb.

Rufian-Henares & De la Cueva[38] estimated the daily HMF intake range between 2.1 and 23 mg per capita. A daily consumption of a bread from the experimental design would therefore contribute to as low as 0.12% of the daily HMF intake to over 54%.

Only one factor has a significant impact on HMF amount in finished breads. That factor is the final baking step and its mean effect represented -158.0 % of overall mean. The breads baked at 250°C for 9 min have systematically higher HMF contents (mean value: 0.79 mg/100g DM) than the breads baked at 200°C for 12 min (mean value: 0.09 mg/100g DM).

The extraction rates of flours, the type of the leavening agent and the prebaking step had no impact on the HMF content. In a study comparing whole-wheat breads to white breads, whole-wheat was shown to reduce the HMF content, 1.7 mg HMF/100g DM for the whole-wheat bread compared to 4.7 mg HMF/100g for the white bread[39]. Higher extraction rates of flours could therefore play a role in reducing HMF contents. But the two extraction rates used for white breads compared in this study did not show any differences in the final HMF content of breads. The leavening agents showed no significant differences either. Such observation can be explained by the multitude of pathways leading to the HMF formation. If sourdough slowed the Maillard reaction by the acidification of the dough,

368 then the lower pH would have favoured the caramelization of sugars and as a consequence the
369 formation of HMF[40]. The prebaking step, moreover, does not seem to affect the HMF content. The
370 mild heat treatment applied during this step aims to generate a stable structure allowing further
371 processing. No browning is observed at this point and we assume therefore that the Maillard reaction
372 and the caramelization have not yet been fully initiated. Furthermore, no interaction between factors
373 was found to be significant.
374 In a previous study, HMF was found to be often related to the lightness parameter L^*, r^2=0.9023, when
375 the same recipe and the same bread-making conditions were used for different baking durations[41]. The
376 same authors found that the two parameters were not significantly correlated when the ingredients and
377 the process varied between the products[37]. The breads of our experimental design fall under the second
378 category since different recipes and different bread-making conditions affecting the colour were
379 applied. This is likely the reason why the linear correlation (r=0.18) between HMF and the lightness
380 parameter L^* is very weak for the 16 breads of the experimental design.
381 Reducing the HMF content in bread is therefore mostly dependent on the final baking step where
382 milder baking conditions generates lower amounts of HMF. However, such mitigation strategies must
383 take into account that the formation pathways of unwanted HMF are the same as for desirable colour
384 and flavour compounds[14].

385 **Thiamine & folate**

386 As can be seen in Table 4, thiamine amounts ranged between 111 and 294 µg/100g DM (75-203
387 µg/100g fresh bread) while folate amounts ranged between 41 and 130 µg/100g DM (27.8-88.5
388 µg/100g fresh bread) in the breads studied. These levels are higher than the ones indicated by Bourre
389 et al.[3] for similar breads where the breads made with the T55 and T65 flours contained 14.6 and 16.8
390 µg of folate /100g respectively and 100 µg of thiamine /100g. The measured amounts are however
391 equivalent to the ones found by Hjortmo et al.[6] for folate levels in white breads (27-139 µg/100g DM)
392 and slightly superior to those indicated by Batifoulier et al.[5] for thiamine amounts in white breads (88-
393 126 µg/100g DM).

The variation of thiamine concentration between the 16 experimental breads was as large as the variation between the repetitions (data not shown). As a result the thiamine level in the 16 types of bread appeared to be unaffected by any of the mean effects and interactions analysed. In a similar way, the variation of folate concentration observed between the experimental breads was slightly larger than the one observed between repetitions. Surprisingly the interaction between the leavening agent, the prebaking and the baking conditions seem to affect the folate contents of breads. Such a case of interaction is rather rare. A confirmation of that 3 factors interaction requires a replication of the experimental design. A replicated design which would take into account the low repeatability would be preferred.

Hjortmo et al.[6] found that the strain and the cultivation conditions of the leavening agent contribute greatly to the final amounts of folate in breads. It can also be expected that the extraction rate of flours would affect the thiamine and folate contents of breads[3]. No similar observations can be made for the current experimental breads since only two white flours were compared.

According to the Regulation (EU) No. 1169/2011, the daily reference intakes (DRI) of thiamine and folate are set at 1100 µg and 200 µg, respectively. One daily portion of the breads tested in this study (170g) would therefore contribute from 11 to 31% and 23 to 75% of the DRI of thiamine and folate, respectively. This is not surprising considering that the main folate source for Italian men is bread [42].

Phytic acid & minerals

Phytic acid levels in the 16 breads, shown in Table 5, varied between 52.45 and 176.4 mg/100g DM (35.44-119.28 mg/100g fresh bread). These amounts are lower than the ones found in the study of Bourre et al.[3] for similar white breads (200 mg/100g). No significant impact of the effects studied was observed in relation to the phytic acid amounts. It is to be noted that, as previously mentioned, phytic acid levels depend on the extraction rate of flours and it is more concentrated in whole grain flours. But since the aim of this study was to compare two white flours, the low amount of phytic acid as well as the lack of observed impact is most likely a result of the absence of wheat husks in the flours.

Similarly the factors studied do not seem to have an impact on the amounts of copper, zinc and iron in the experimental breads. Manganese levels are, however, affected by the extraction rate, the leavening agent and the baking conditions. Calcium levels are affected by the same factors as for manganese as well as the interaction between the leavening agent and the prebaking conditions. Phosphorus, magnesium, potassium, calcium and manganese levels are affected by the interaction between the extraction rate and the baking conditions. Potassium levels are also affected by the baking conditions and the interaction between the prebaking and the baking conditions. While sometimes statistically significant, the variation of a mineral concentration was never very important (mean effect <8%). This indicates weak effects of the four factors on the final mineral content of bread.

Among the four factors, the extraction rate of the flour and the baking conditions seem to be the most influential factors affecting the minerals levels in the studied breads. A T65 flour and a baking at 200°C for 12 min seem to increase certain mineral levels without increasing the anti-nutritional phytic acid. Extraction rates of flours are known to influence minerals levels. A previous study showed that cooking conditions also affect mineral levels in foods[43]. Cooking conditions influence the chemical interactions within a food matrix and thus could cause complexation of certain minerals rendering them difficult to extract and analyse. Using a combination of yeast and sourdough as leavening agents improved calcium and manganese amounts in the finished breads A previous study noted that a prolonged fermentation with sourdough leads to improved magnesium and phosphorus solubility due to acidity[25]. Thus, as we observed in the analysis of the current experimental design, the mineral content of breads is dependent on multiple factors.

Selection of the optimal conditions among the sixteen combinations tested

It can be stated in conclusion that various factors affected the chemical quality of white breads. CML was mostly affected by the baking factor but also by the leavening agent. HMF was affected by the baking factor alone. Thiamine and folate were not affected by any of the factors analysed. The same applies to phytic acid and certain minerals like copper, zinc and iron. The remaining minerals were

mostly affected by the extraction rate and the baking of the breads but also by the leavening agent and various interactions between the four factors studied. All these results showed that both formulation and process are implicated in the final quality of white breads. Thus improving one independently of the other is not sufficient for an optimal final product.

Figure 1 shows two radar charts which expose how six breads of the experimental design compare to the target optimal contents of minerals, vitamins, phytic acid and MRPs. Figure 1 A represents three breads with the most optimal results relative to the target bread, while Figure 1 B represents the less optimal results relative to the target bread. Standardized data are presented in this figure. The value 1 corresponds to the maximal value measured in the experimental breads for the compound in question and 0.1 to the minimal value measured, while 0 corresponds to the absence of the compound in question. The B01, B09 and B13 samples seem to combine the conditions providing the most optimal results as they maximize mineral contents and minimize MRP contents (Fig. 1-A). These three breads have in common the highest extraction rate (T65) of the study and the "mildest" baking conditions (12 min, 200°C) but have different combinations of leavening agents and pre-baking conditions. Two of these breads are made using sourdough (B01 and B09).

As it is presented in Tables 3 and 4 and illustrated in Figure 1, the bread B01, which was close to the optimal/target bread, contained 5 times less HMF, 28% less CML, 26% more vitamin B1, and only 11% less vitamin B9 compared to the bread B02 which was among the less optimal breads. The same comparison can be made with breads B13 and B14. In other words, our study indicates that among the 16 combinations tested, we were able to find the optimum conditions to decrease significantly unwanted neoformed compounds such as HMF and CML without compromising the nutritional quality of bread. Even though reading the individual data might give the impression that some formulation and process parameters are favourable conditions for an optimum concentration of vitamins B1 and B9, the statistical analysis rejected this observation.

Overall, when only T65 flours are available, selecting the optimal formulation and baking conditions used either for samples B01, B09 or B13 should provide breads with a satisfactory nutritional quality

(*e.g.* thiamine: 118 to 175 µg/100g; folate: 43 to 65 µg/100g) and low amounts of CML (<0.47µg/100g) and HMF (<126µg/100g).

For the less satisfying breads presented in Figure 1-B, all three were prepared using the more severe baking conditions (9 min, 250°C) and yeast as the leavening agent. However, the extraction rate of flour and the pre-baking conditions varied. These observations further illustrate the importance of the combination of both formulation and process factors on their impacts on the nutritional quality of breads.

For a complete evaluation of the chemical and nutritional quality of breads, other compounds should also be taken into consideration. To this purpose, the analysis of MRPs with positive health impacts such as the melanoidins is under detailed investigation in our laboratory.

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564

565 **Table 1:** The 2-level full factorial experimental design applied for bread-making.

566 **Table 2:** Dry content, crust/whole bread ratio, crude protein and lysine contents of the breads studied
567 (data are means ± SD)

568 **Table 3:** MRPs amounts and colorimetric measures of the breads studied (data are means ± SD)

569 **Table 4:** Thiamine and folate amounts in the breads studied (data are means ± SD)

570 **Table 5:** Minerals and phytic acid amounts in the breads studied (data are means ± SD)

571 **Figure 1:** A comparison between three breads with the most optimal results (A) and three breads with
572 the less optimal results (B) relative to the optimal bread. The value 1 corresponds to the maximal value
573 measured in the experimental breads and 0.1 to the minimal value measured. The value 0 corresponds
574 to the absence of the compound in question.

575

Table 1:

Name	Extraction rate	Leavening agent	Pre-baking	Baking
B01	T65	Yeast + Sourdough	13 min; 130°C	12 min; 200°C
B04	T55	Yeast + Sourdough	4 min; 250°C	12 min; 200°C
B06	T65	Yeast	13 min; 130°C	12 min; 200°C
B08	T55	Yeast	4 min; 250°C	12 min; 200°C
B09	T65	Yeast + Sourdough	4 min; 250°C	12 min; 200°C
B13	T65	Yeast	4 min; 250°C	12 min; 200°C
B15	T55	Yeast	13 min; 130°C	12 min; 200°C
B16	T55	Yeast + Sourdough	13 min; 130°C	12 min; 200°C
B02	T65	Yeast	4 min; 250°C	9 min; 250°C
B03	T65	Yeast + Sourdough	4 min; 250°C	9 min; 250°C
B05	T65	Yeast + Sourdough	13 min; 130°C	9 min; 250°C
B07	T65	Yeast	13 min; 130°C	9 min; 250°C
B10	T55	Yeast + Sourdough	13 min; 130°C	9 min; 250°C
B11	T55	Yeast	13 min; 130°C	9 min; 250°C
B12	T55	Yeast + Sourdough	4 min; 250°C	9 min; 250°C
B14	T55	Yeast	4 min; 250°C	9 min; 250°C

Table 2:

Breads	Dry content (g/100g)	Crust/Bread Ratio (%)	Crude Protein content of breads (g/100g DM)	Lysine (g/100g DM)
B01	66.29 ± 0.24	42.53 ± 1.81	12.62 ± 1.08	0.43 ± 0.01
B02	68.08 ± 0.07	39.50 ± 4.29	12.43 ± 0.51	0.40 ± 0.01
B03	67.11 ± 0.37	41.59 ± 3.63	11.81 ± 0.50	0.43 ± 0.03
B04	65.55 ± 0.15	40.83 ± 2.96	12.70 ± 0.42	0.39 ± 0.06
B05	68.10 ± 0.17	48.17 ± 4.81	12.49 ± 0.22	0.42 ± 0.03
B06	66.66 ± 0.09	42.10 ± 1.86	12.16 ± 0.37	0.42 ± 0.01
B07	67.62 ± 0.25	40.75 ± 3.35	13.30 ± 0.17	0.39 ± 0.01
B08	67.21 ± 0.23	37.60 ± 5.81	12.02 ± 0.96	0.41 ± 0.00
B09	65.31 ± 0.17	31.98 ± 4.17	12.11 ± 0.84	0.44 ± 0.01
B10	70.90 ± 0.23	39.19 ± 3.04	13.22 ± 0.69	0.42 ± 0.02
B11	69.08 ± 0.54	36.95 ± 3.53	13.68 ± 0.49	0.38 ± 0.01
B12	68.99 ± 0.49	40.87 ± 8.66	12.43 ± 0.73	0.40 ± 0.01
B13	65.23 ± 0.23	30.17 ± 3.60	12.56 ± 1.10	0.43 ± 0.01
B14	67.75 ± 0.47	35.79 ± 2.24	12.98 ± 0.66	0.38 ± 0.01
B15	67.57 ± 0.51	31.22 ± 5.77	12.53 ± 0.72	0.41 ± 0.01
B16	68.15 ± 0.25	30.98 ± 3.69	11.88 ± 0.35	0.42 ± 0.01

Table 3:

Breads	HMF (mg/100g DM)	CML (mg/100g DM)	CML (mg /kg proteins)	CML (mmol /mol lysine)	*L**
B01	0.19 ± 0.01	0.70 ± 0.05	55.54 ± 4.27	1.17 ± 0.09	67.28 ± 2.62
B02	0.92 ± 0.01	0.97 ± 0.1	78.36 ± 7.25	1.74 ± 0.16	65.88 ± 2.24
B03	0.81 ± 0.03	0.81 ± 0.04	68.41 ± 5.17	1.34 ± 0.10	65.71 ± 2.93
B04	0.13 ± 0.01	0.70 ± 0.01	54.75 ± 1.00	1.28 ± 0.02	65.70 ± 3.84
B05	0.83 ± 0.05	0.81 ± 0.03	64.85 ± 2.23	1.38 ± 0.05	63.99 ± 2.12
B06	0.02 ± 0.00	0.75 ± 0.1	61.93 ± 7.83	1.28 ± 0.16	67.60 ± 3.12
B07	0.57 ± 0.05	0.92 ± 0.09	69.14 ± 6.03	1.69 ± 0.15	66.53 ± 1.79
B08	0.08 ± 0.01	0.75 ± 0.04	61.99 ± 3.42	1.30 ± 0.07	71.33 ± 1.63
B09	0.12 ± 0.01	0.70 ± 0.02	57.56 ± 1.64	1.13 ± 0.03	68.75 ± 2.80
B10	0.94 ± 0.08	0.82 ± 0.07	61.95 ± 6.47	1.40 ± 0.15	65.02 ± 2.09
B11	0.59 ± 0.02	0.93 ± 0.05	67.97 ± 4.61	1.75 ± 0.12	68.14 ± 2.93
B12	0.89 ± 0.08	0.71 ± 0.06	57.18 ± 5.05	1.27 ± 0.11	63.58 ± 1.31
B13	0.11 ± 0.01	0.72 ± 0.04	57.51 ± 3.43	1.20 ± 0.07	70.28 ± 2.78
B14	0.75 ± 0.01	0.94 ± 0.12	72.69 ± 8.65	1.78 ± 0.21	66.51 ± 2.42
B15	0.03 ± 0.00	0.74 ± 0.02	58.80 ± 2.78	1.29 ± 0.06	63.54 ± 2.68
B16	0.05 ± 0.00	0.75 ± 0.04	63.51 ± 3.10	1.29 ± 0.06	64.43 ± 2.86

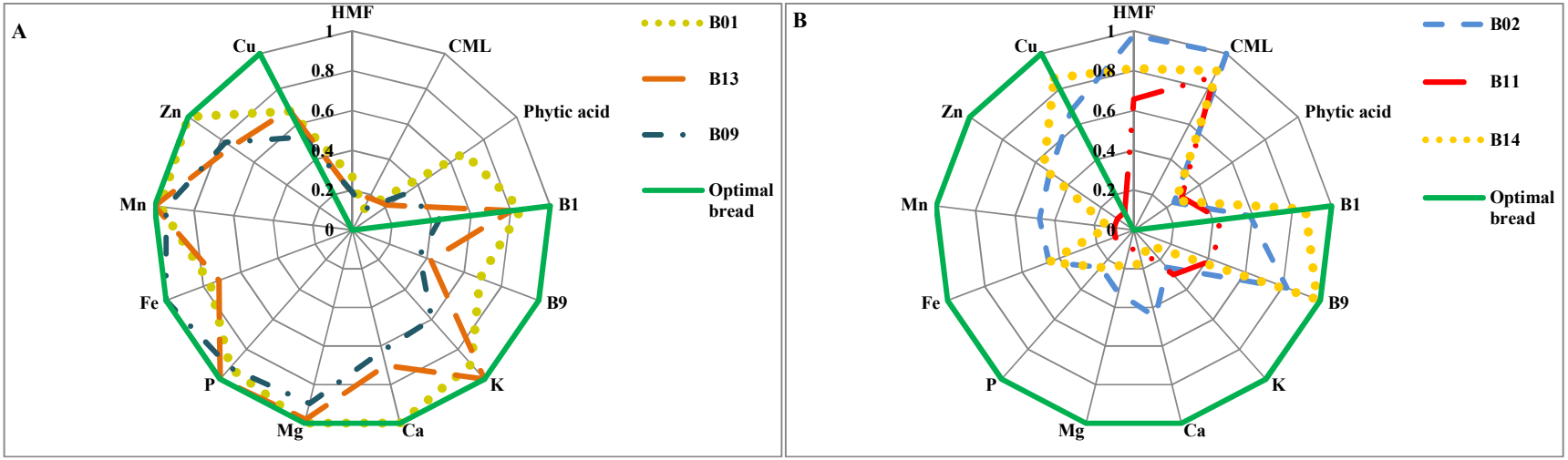
Table 4:

Breads	Thiamine (µg/100g DM)			Folate (µg/100g DM)		
B01	264.01	±	102.41	98.81	±	24.70
B02	210.04	±	39.47	112.22	±	28.05
B03	236.91	±	50.57	96.70	±	24.17
B04	238.00	±	72.28	116.86	±	29.22
B05	286.36	±	59.19	129.96	±	32.49
B06	246.02	±	108.20	111.91	±	27.98
B07	230.71	±	73.20	72.02	±	18.01
B08	203.84	±	142.03	67.70	±	16.92
B09	180.67	±	21.65	66.60	±	16.65
B10	214.38	±	32.91	72.92	±	18.23
B11	179.49	±	15.35	73.10	±	18.27
B12	294.24	±	60.47	59.43	±	14.86
B13	254.48	±	68.29	72.51	±	18.13
B14	267.16	±	17.74	128.41	±	32.10
B15	111.00	±	34.99	59.64	±	14.91
B16	158.48	±	10.38	40.79	±	10.20

Table 5:

Breads	K (mg/100g DM)	Ca (mg/100g DM)	Mg (mg/100g DM)	P (mg/100g DM)	Fe (mg/100g DM)	Mn (mg/100g DM)	Zn (mg/100g DM)	Cu (mg/100g DM)	Phytic Acid (mg/100g DM)
B01	205 ± 21	28.70 ± 2.87	37.8 ± 3.78	157 ± 7.85	1.57 ± 0.24	1.47 ± 0.15	1.25 ± 0.13	0.409 ± 0.08	132.45 ± 7.25
B02	192 ± 19	26.50 ± 2.65	34.5 ± 3.45	148 ± 7.40	1.44 ± 0.22	1.30 ± 0.13	1.12 ± 0.11	0.410 ± 0.08	72.35 ± 5.95
B03	189 ± 19	25.90 ± 2.59	34.4 ± 3.44	146 ± 7.30	1.60 ± 0.24	1.34 ± 0.13	1.10 ± 0.11	0.354 ± 0.07	87.70 ± 3.50
B04	198 ± 20	26.10 ± 2.61	36.0 ± 3.60	153 ± 7.65	1.46 ± 0.22	1.32 ± 0.13	1.12 ± 0.11	0.408 ± 0.08	70.50 ± 12.60
B05	195 ± 20	26.20 ± 2.62	34.5 ± 3.45	148 ± 7.40	1.47 ± 0.22	1.31 ± 0.13	1.11 ± 0.11	0.496 ± 0.10	86.45 ± 13.55
B06	193 ± 19	26.00 ± 2.60	34.6 ± 3.46	148 ± 7.40	1.46 ± 0.22	1.31 ± 0.13	1.15 ± 0.11	0.343 ± 0.07	65.75 ± 11.95
B07	202 ± 20	27.00 ± 2.70	36.8 ± 3.68	156 ± 7.80	1.44 ± 0.22	1.35 ± 0.14	1.15 ± 0.11	0.328 ± 0.07	176.40 ± 13.10
B08	200 ± 20	26.40 ± 2.64	36.4 ± 3.64	154 ± 7.70	1.45 ± 0.22	1.33 ± 0.13	1.12 ± 0.11	0.322 ± 0.06	79.00 ± 19.60
B09	199 ± 20	27.20 ± 2.72	37.3 ± 3.73	157 ± 7.85	1.67 ± 0.25	1.46 ± 0.15	1.19 ± 0.12	0.370 ± 0.07	82.35 ± 25.05
B10	194 ± 19	26.50 ± 2.65	34.8 ± 3.48	148 ± 7.40	1.42 ± 0.21	1.31 ± 0.13	1.08 ± 0.11	0.309 ± 0.06	90.70 ± 6.60
B11	193 ± 19	25.10 ± 2.51	33.4 ± 3.34	146 ± 7.30	1.29 ± 0.19	1.17 ± 0.12	1.01 ± 0.10	0.256 ± 0.05	78.90 ± 2.10
B12	200 ± 20	26.80 ± 2.68	36.7 ± 3.67	156 ± 7.80	1.43 ± 0.21	1.34 ± 0.13	1.12 ± 0.11	0.344 ± 0.07	74.15 ± 6.65
B13	207 ± 21	27.50 ± 2.75	37.7 ± 3.77	158 ± 7.90	1.55 ± 0.23	1.48 ± 0.15	1.18 ± 0.12	0.411 ± 0.08	68.85 ± 21.95
B14	189 ± 19	25.40 ± 2.54	33.8 ± 3.38	148 ± 7.40	1.44 ± 0.22	1.18 ± 0.12	1.13 ± 0.11	0.459 ± 0.09	74.10 ± 7.50
B15	195 ± 20	25.80 ± 2.58	34.0 ± 3.40	148 ± 7.40	1.40 ± 0.21	1.18 ± 0.12	1.10 ± 0.11	0.394 ± 0.08	52.45 ± 10.95
B16	195 ± 20	25.90 ± 2.59	34.0 ± 3.40	148 ± 7.40	1.48 ± 0.22	1.20 ± 0.12	1.21 ± 0.12	0.429 ± 0.09	65.45 ± 3.25

Figure 1:



Graphical Abstract: