

Evaluation of antioxidant capacity and formation of processing contaminants during rye bread making

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SUMMARY

The formation of antioxidants and contaminants such as acrylamide (ACR), hydroxymethylfurfural (HMF) and furfural (FUR) during rye bread making was investigated. Flours with extraction rates of 700, 850, 950 and 1000 g·kg⁻¹ were used. Flour, dough, slice of bread, crust and crumb were analysed. Results are expressed on dry matter basis. Antioxidant capacity of water- and lipid-soluble antioxidants were measured by photochemiluminescence assay. Quencher approach was adapted to total antioxidant capacity and total reducing capacity (Folin-Ciocalteu) methods. Bread based on wholemeal flour showed the highest antioxidant capacity and the lowest levels of potentially harmful compounds (ACR: 50 µg·kg⁻¹; HMF: 33.6 mg·kg⁻¹; FUR: 1.6 mg·kg⁻¹). Estimation of dietary intake of ACR, HMF and FUR from rye bread was 4.93 ng·kg⁻¹·per day, 4.47 µg·kg⁻¹·per day and 0.18 µg·kg⁻¹·per day, respectively. Levels of ACR, HMF and FUR were 27-fold, 631-fold and 31-fold in crust, respectively, which represents less than 15% of the total weight of the bread, compared to crumb. Crust was also the principal contributor to maintain the overall water-soluble antioxidant capacity being up to 3.5-fold more active than crumb or dough. Any mitigation strategy might consider risk and benefits associated with crust.

Keywords

antioxidant capacity; acrylamide; hydroxymethylfurfural; furfural; rye bread

Traditional foods play a pivotal role as they reflect cultural inheritance and influence the dietary patterns in different countries. Nowadays, consumers are more interested in nutritional and health aspects of traditional foods and more attention is being drawn by manufacturers to these requirements [1]. Traditional rye bread making includes three main stages: sourdough preparation (by mixing sour, flour, water, yeasts and salt), dough fermentation and baking. Baking induces a series of physical and chemical alterations including evaporation of water, volume expansion and formation of porous structure, protein denaturation, starch gelatinization and also changes on the surface of the formed bread [2]. The most visible transformation is connected with colour development in the crust due to chemical reactions occurring during baking. Reactions involved in this process are essentially Maillard reaction and

caramelization [3], which influence the flavour strongly reflecting to the consumer's acceptability of products [4]. Other food quality parameter such as the nutritional value is also affected by the impairment of available amino acid caused by Maillard reaction.

Hydroxymethylfurfural (HMF), furfural (FUR) and acrylamide (ACR), within others, have been reported as newly formed potentially harmful compounds and, as a consequence, they are used as index of food quality deterioration [5–9]. A huge number of attempts have been made to mitigate the formation of those compounds in foodstuffs with special attention to ACR [10, 11]. Formation of ACR occurring during manufacturing of bakery products has received great attention in the recent years and mitigation strategy is of European concern. Among the mitigation strategies, the addition of glycine and the use of the en-

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zyme asparaginase seem the most promising [12, 13]. On the other hand, the addition of antioxidant compounds or extracts of plant origin is currently under investigation, and preliminary results published until now are extremely promising [14, 15], although some controversial effect was observed in model systems [16]. Simultaneously, health-supporting compounds, such as antioxidants, are also generated during baking [17]. It has been reported that colour development induced during baking, which is attributed to the formation of advanced products derived from both Maillard and caramelization reactions, strongly correlated with contents of compounds possessing antioxidant capacity [18]. For instance, melanoidins are able to scavenge peroxy, hydroxyl, DPPH• (2,2-diphenyl-1-picrylhydrazyl) and ABTS•⁺ (2,2'-azobis-3-ethylbenzothiazoline-6-sulphonic acid) radicals [19–22], to chelate metal ions [19, 23], to prevent oxidative stress in cellular cell lines [24] and to modulate the phase I and II xenobiotic enzymatic machinery for chemopreventive response [25]. Thus, changes in the technological process and/or in the bread recipe should be considered both for obtaining healthier foods and mitigation of harmful compound [26].

To the best of our knowledge, no data on the formation of process contaminants and beneficial products associated with antioxidant capacity during rye bread making according to the traditional procedure have been investigated, which was the aim of the present research.

MATERIALS AND METHODS

Chemicals

Hydroxymethylfurfural, potassium ferrocyanide, zinc acetate, potassium persulfate, acrylamide, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and ferulic acid were supplied by Sigma-Aldrich (Diesenhofen, Germany). Furfural was purchased from Fluka (Schnelldorf, Germany). Acetonitrile of liquid chromatography grade was supplied by Scharlab (Madrid, Spain). Cellulose, sodium carbonate and ethanol were from POCh (Gliwice, Poland). [¹³C₃]-Acrylamide of isotopic purity was from Cambridge Isotope Labs (Andover, Massachusetts, USA). ACW kit No. 400.801 (Antioxidant Capacity of Water soluble substance) and ACL kit No. 400.803 (Antioxidant Capacity of Lipid soluble substance) were from Analytic-Jena (Jena, Germany). Ultrapure

water was used throughout the experiments (Simplicity System, Millipore, Molsheim, France). All other chemical were of analytical grade.

Materials

Rye grains of the cultivar Warko were selected from breeding materials grown in central Poland (DANKO, Plant Breeding, Laski, Poland) in 2007, tempered to 14.0% moisture and milled on a Quadrumat Senior laboratory mill (Brabender, Germany) to obtain flour with extraction rates of 1000 g·kg⁻¹ (wholemeal flour), 950 g·kg⁻¹ (brown flour), 850 g·kg⁻¹ (brown flour) and 700 g·kg⁻¹ (light flour). The flours obtained were used for rye bread making in a pilot-scale bakery.

Rye bread making process

Rye bread type I was based on wholemeal flour, type II on flour with extraction rate of 950 g·kg⁻¹, type III on flour with extraction rate of 850 g·kg⁻¹ and type IV on light flour with extraction rate of 700 g·kg⁻¹. Salt and baker's yeast used in the formulation of bread dough were purchased from a local food manufacturer. Rye breads were produced in Poland at a pilot-scale bakery using traditional sourdough fermentation with baker's yeast addition. For each type of bread the sourdough starter was prepared from the respective type of flour. The three-stage method was used to make dough. Sourdough starter, as the first stage, was prepared by mixing 36% of the respective rye flour and 64% of water. This mixture was left to ferment for 48 h at 28 °C. The second stage – sour was prepared by mixing 300 g of sourdough starter, 300 g of each type of rye flour, 300 ml of water and 10 g of yeast. The mixture was left to ferment for 3 h at 28 °C. In the third stage, dough was prepared by mixing 800 g of sour, 600 g of the respective rye flour, 300 g of water and 20 g of salt, and then the dough was left for final fermentation for 30 min at 28 °C in a ferment chamber. Pieces of dough (350 g) were molded by hand, panned and proofed for 45 min at 28 °C (75% relative humidity). Breads were baked in an electric oven at 260 °C for 40 min. Breads were cut into slices of 1 cm thickness and crumbs and crusts were manually separated. The samples were frozen, lyophilized and ground. Powdered samples (flour, dough, bread and the corresponding crumbs and crusts) were stored at 4 °C in hermetic bags sealed under vacuum. The dry matter contents in slice, crumb and crust of bread based on rye flours with extraction rates of 1000, 950, 850 and 700 g·kg⁻¹ were of 626, 630, 641 and 545 g·kg⁻¹ in slices, 604, 547, 617 and 608 g·kg⁻¹ in crumbs, and 893, 895, 888 and 892 g·kg⁻¹ in crusts, respectively.

Determination of acrylamide (ACR)

ACR was analysed according to RUFIÁN-HE-NARES et al. [27]. Four hundred and fifty mg of powdered samples were weighed and suspended in 5 ml of milli-Q water. Subsequently, 100 μ l of 10 mg·l⁻¹ [¹³C₃]-labelled acrylamide methanol solution was added into sample suspension and then thoroughly mixed for 1 min using a vortex. After that, 750 μ l of Carrez I and 750 ml of Carrez II were added, and the mixture was vortexed for 10 s. Then the sample was left for 10 min at room temperature and finally centrifuged at 4 °C for 15 min at 2400×g (Hettich Universal-320R, Tuttingen, Germany). Supernatants were transferred to separate microtubes and were frozen. Before acrylamide analysis, samples were defrosted and centrifuged at 10000×g at room temperature for 10 min (Hettich Mikro-20). Aliquots of 1 ml of the supernatants were clarified on a pre-conditioned Oasis HLB cartridge (Waters, Milford, Massachusetts, USA). The first seven drops were discarded and the rest was collected in glass vials for further LC-MS analysis. LC-MS was performed on an Agilent 1100 liquid chromatograph coupled to an Agilent Quadrupole MS detector (Agilent Technologies, Palo Alto, California, USA). Separation was carried out on an Inertsil ODS-3V analytical column (250 × 4.6 mm, 5 μ m; GLC Sciences, Tokyo, Japan) at 32 °C with isocratic elution. The injection volume was 60 μ l, the mobile phase was water: formic acid (99.8:0.2; v/v) and a flow rate was of 0.6 ml·min⁻¹. Data acquisition was performed, with a delay time of 8 min, in a selected ion monitoring mode (SIM). Monitored ions were those of *m/z* of 72.1 and 75.1 for ACR and ¹³C₃-ACR, respectively. ACR was quantified using a calibration curve constructed by injecting external standard solutions of ACR in concentrations ranging from 1 μ g·l⁻¹ to 100 μ g·l⁻¹. The results were expressed as μ g·kg⁻¹ (micrograms of ACR per kilogram of dry matter). The method was in-house validated for linearity, precision and recovery. Accuracy was externally accredited by proficiency tests launched by the Institute for Reference Materials and Measurements (Geel, Belgium) and FAPAS (Food Analysis Performance Assessment Scheme), yielding satisfactory *z*-score of -0.5, -1.0 and -1.1. The analyses are integrated within the scope of a certified laboratory controlled by AENOR (Spanish Association for Standardisation and Certification).

Determination of hydroxymethylfurfural (HMF) and furfural (FUR)

HMF and FUR were chromatographically determined in samples according to RUFIÁN-HE-NARES et al. [28]. Briefly, 0.5 g of powdered

sample was weighed and added to 5 ml of 0.1% formic acid, and the mixture was vortexed for 10 s. Then, the sample was clarified with 250 μ l of Carrez I and 250 μ l of Carrez II solutions. After that step, the mixture was centrifuged at 2400×g for 10 min at 4 °C (Hettich Universal-320R), and the supernatant was collected. The extraction procedure was repeated twice and finally the supernatants were pooled, divided in aliquots of 1 ml and filtered employing nylon filters of the pore size of 0.45 μ m. The filtrates were used for analysis of HMF and FUR. Results were expressed as mg·kg⁻¹ (milligrams of HMF or FUR per kilogram of dry matter).

Antioxidant capacity by photochemiluminescence (PCL) assay

Photochemiluminescence (PCL) assay, based on the methodology of POPOV and LEWIN [29], was used to measure the antioxidant capacity of the extracts with a Photochem apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photosensitizer, when exposed to UV light. The assay was performed using both ACW and ACL kits provided by the manufacturer to measure the antioxidant capacity of hydrophilic and lipophilic compounds, respectively. The experimental procedure was recently described in detail by ZIELIŃSKA et al. [30]. Prior to analysis, hydrophilic and lipophilic antioxidants were extracted from the samples. For extraction of the hydrophilic antioxidants, 100 mg of the sample were added to 1 ml of deionized water treated in an ultrasonic bath for 30 s, vortexed for 30 s and centrifuged at 13200×g for 5 min at 4 °C (Centrifuge 5415R; Eppendorf, Hamburg, Germany). Water extraction was repeated five times and the supernatants were pooled into calibrated flasks. The same procedure was applied to extraction of lipophilic compounds with ethanol/hexane (4:1, v/v). The freshly prepared supernatants were taken for further evaluation of the antioxidant capacity. The extracts for ACW and ACL measurements were centrifuged at 16000×g for 5 min prior to analysis. The assay was carried out in triplicate, and the antioxidant capacity was expressed as mmol·kg⁻¹ (Trolox equivalents per kilogram of dry matter).

Total antioxidant capacity by direct ABTS assay

The direct measurement of the total antioxidant capacity was performed according to GÖKMEN et al. [31] with some modifications outlined below. The ABTS stock solution was prepared according to RE et al. [32] and then it was diluted by 50% aqueous ethanol. The freshly prepared ABTS

working solution (6 ml) was directly added to the powdered samples (10 mg) followed by continuous stirring for 30 min at room temperature without light exposition. After that time, samples were centrifuged at room temperature at $13\,200\times g$ for 10 min and the absorbance of optically clear supernatants was measured at 734 nm using a spectrophotometer (UV-160 1PC; Shimadzu, Kyoto, Japan). For the sample dilution as well as for blanks and standards, solid-state cellulose was used. A calibration curve of Trolox in the range of concentrations of $0.125\text{ mmol}\cdot\text{l}^{-1}$ to $1.75\text{ mmol}\cdot\text{l}^{-1}$ was constructed. All measurements were carried out in triplicate and were expressed as $\text{mmol}\cdot\text{kg}^{-1}$ (as Trolox equivalents per kilogram of dry matter). The direct analysis of the solid samples has been called as “quencher approach” by its authors [31].

Total reducing capacity (TRC) by direct assay

The “quencher approach” was adapted for first time to the Folin-Ciocalteu reaction in order to achieve a direct measurement of the reducing capacity of the samples without any extraction step prior to analysis and without underestimation of this activity. The proposed method is based on the reaction of Folin-Ciocalteu reagent with the functional hydroxy groups of free, chemically bound and physically entrapped phenolic compounds. Folin-Ciocalteu phenol reagent consists of a mixture of the heteropoly acids, phosphomolybdic and phosphotungstic acids, in which the molybdenum and tungsten are in the 6^+ state. On reduction with certain reducing agents, so called molybdenum blue and tungsten blue are formed, in which the mean oxidation state of the metals is between 5 and 6. As the reduced Folin-Ciocalteu reagent is blue, it is possible to carry out the determination of the total reducing capacity of solid sample with a spectrophotometer in the wavelength range from 500 nm to 750 nm. The assay was carried out as follows: $250\text{ }\mu\text{l}$ of Folin-Ciocalteu reagent prepared with 50% aqueous ethanol were directly added to 30 mg of individual powdered samples. Then, 0.5 ml of saturated Na_2CO_3 was added and the mixture was mixed well on vortex followed by addition of 4 ml of 50% ethanol. Next, the mixture was left for 25 min in the dark at room temperature. After that time, the mixture was centrifuged at $13\,200\times g$ for 10 min at room temperature. The absorbance readings were taken at $\lambda = 725\text{ nm}$ using a spectrophotometer (UV-160 1PC). The ferulic acid prepared in 50% aqueous ethanol was used for the construction of the calibration curve within the concentration range of $0.062\text{--}1.0\text{ g}\cdot\text{l}^{-1}$. All measurements were performed in triplicate. The total reducing capacity (TRC) was expressed

as $\text{g}\cdot\text{kg}^{-1}$ (ferulic acid equivalents per kilogram of dry matter).

Statistical analysis

Statistical significance of data was analysed by one-way analysis of variance (ANOVA) followed by the Fischer LSD test using the Statistica 7.0 Program (StatSoft, Tulsa, Oklahoma, USA) for Windows. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Potential health-promoting properties of rye breads

Scavenging activity of superoxide anion radicals

PCL assay was carried out to assess the antioxidant capacity of both lipophilic and hydrophilic compounds from rye bread samples. Lipophilic compounds were extracted with organic solvents and all the samples were found to be able to scavenge superoxide anion radicals to some extent (Fig. 1). Not only the extraction rate of the flour, but also the dough preparation step had a significant impact on the antioxidant capacity exerted by the lipophilic fraction. Dough formulated from wholemeal and $950\text{ g}\cdot\text{kg}^{-1}$ extraction rate showed similar PCL-ACL values, which were higher compared to those found for the rest of the samples hereby studied. Therefore, it is plausible that ethanol/hexane soluble antioxidants could be released during dough fermentation, as it was suggested by LIUKKONEN et al. [33]. The PCL-ACL values of rye flours ranged from $1.10\text{ mmol}\cdot\text{kg}^{-1}$ to $1.65\text{ mmol}\cdot\text{kg}^{-1}$ and the lowest PCL-ACL activity was recorded for rye flour with the extraction rate of $700\text{ g}\cdot\text{kg}^{-1}$.

After baking, a significant decrease in the PCL-ACL values by approximately 60% was observed. The PCL-ACL values of the crumb were higher than those found in crust, and both significantly decreased with lower flour extraction rate (Fig. 1). The antioxidant capacity of newly formed lipid-soluble antioxidants originating from crust were lower (11.9, 23.5, 15.1 and 12.5%, respectively) in comparison to those obtained for slices based on flour extraction rate (1000, 950, 850 and $700\text{ g}\cdot\text{kg}^{-1}$, respectively). Contrary to that observed for the potentially harmful process contaminants and discussed later, crumb exerted a greater contribution to the overall antioxidant capacity towards lipid-soluble antioxidants than crusts in bread slices.

The antioxidant capacity of the aqueous extracts (PCL-ACW) is shown in Fig. 2. Hydrophilic

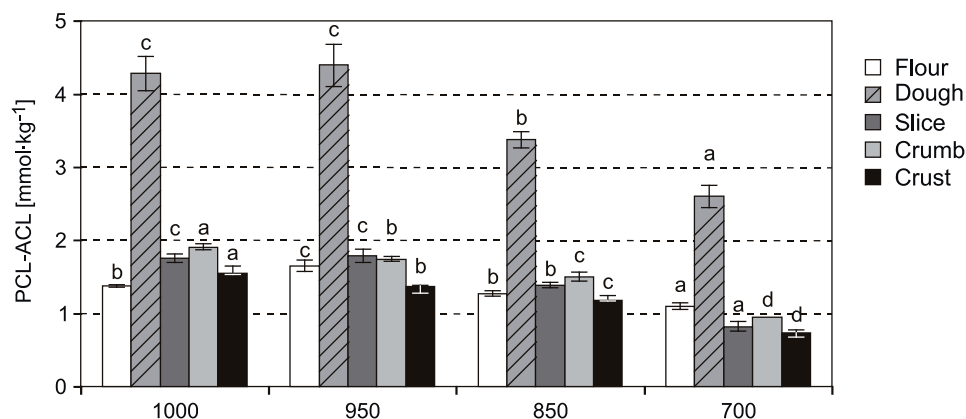


Fig. 1. Antioxidant capacity of the four types of rye flour, dough and bread (including slice, crumb and crust) formed by lipid-soluble compounds (PCL-ACL).

Data are expressed on a dry matter basis. Vertical bars represent the standard deviation. Different letters within series (flour, dough, slice, crumbs and crust) are statistically different ($P < 0.05$). Extraction rates of flours in horizontal layer are expressed as $\text{g}\cdot\text{kg}^{-1}$.

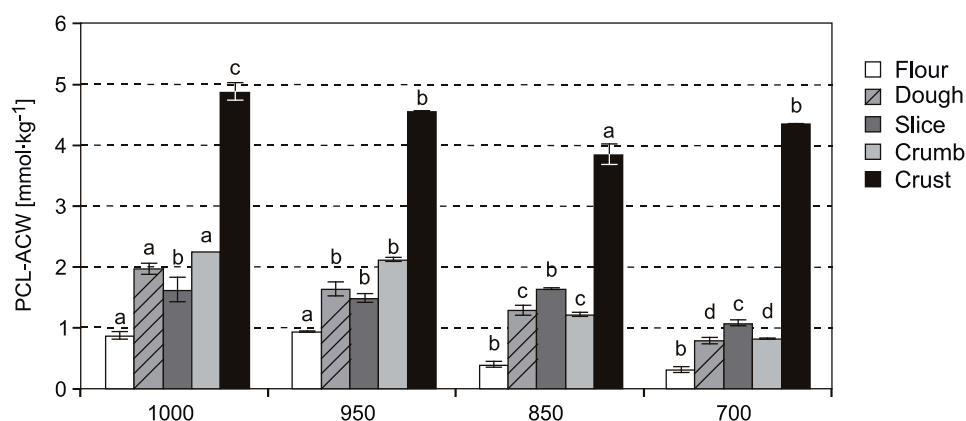


Fig. 2. Antioxidant capacity of the four types of rye flours, doughs and breads (including slices, crumbs and crusts) formed by water-soluble compounds (PCL-ACW).

Data are expressed on a dry matter basis. Vertical bars represent the standard deviation. Different letters within series (flour, dough, slice, crumbs and crust) are statistically different ($P < 0.05$). Extraction rates of flours in horizontal layer are expressed as $\text{g}\cdot\text{kg}^{-1}$.

compounds present in rye flour with different extraction rates were able to scavenge superoxide anion radicals to some extent. The highest scavenging effect was noted for wholemeal flour and flour with extraction rate of $950 \text{ g}\cdot\text{kg}^{-1}$, whereas the light flour showed the lowest ability to scavenge superoxide anion radicals. This relationship was in agreement with our previous work on the bioactive compounds in rye flours with different extraction rates [34]. The dough preparation step caused a significant increase in the antioxidant capacity exerted by the hydrophilic fraction when compared to flours. The ability of hydrophilic fractions to scavenge superoxide anion radicals was more than two-fold higher than it was noted for flours.

Moreover, almost linear decrease in PCL-ACW values of doughs was noted when formulated on rye flours with the extraction rate of 1000, 950, 850 and $700 \text{ g}\cdot\text{kg}^{-1}$, respectively. The PCL-ACW values of doughs ranged from $1.96 \text{ mmol}\cdot\text{kg}^{-1}$ to $0.78 \text{ mmol}\cdot\text{kg}^{-1}$ (Trolox equivalents per kilogram of dry matter) in this case. Baking affected the antioxidant capacity of the samples due to water soluble antioxidants. A major formation of water-soluble antioxidants (ACW) was found in crusts. The composition of the flour influenced the values on antioxidant capacity of the dough. However, no statistically significant differences were noted between slices prepared from bread based on flour with extraction rates of 1000, 950 and $850 \text{ g}\cdot\text{kg}^{-1}$.

Breads made on the basis of refined flours (extraction rate of $700 \text{ g}\cdot\text{kg}^{-1}$) contained lower amounts of water-soluble antioxidants than those less treated (extraction rates of 1000, 950 and 850 $\text{g}\cdot\text{kg}^{-1}$).

Total antioxidant capacity (TAC)

Results on total antioxidant capacity of the samples measured by direct contact of the solid samples with the $\text{ABTS}^{\bullet+}$ radicals are shown in Fig. 3. Similar TAC values were obtained for samples corresponding to flours with extraction rates of 1000 $\text{g}\cdot\text{kg}^{-1}$ and 950 $\text{g}\cdot\text{kg}^{-1}$, respectively. In samples submitted to further extraction (850 $\text{g}\cdot\text{kg}^{-1}$ and 700 $\text{g}\cdot\text{kg}^{-1}$ extraction rates), significantly lower amounts of scavengers of $\text{ABTS}^{\bullet+}$ radicals were detected. TAC values of the dough were lower than those detected in its corresponding flours;

however, the differences between both types of samples were not statistically significant. According to the comparative analysis of the TAC values found by analysis of dough and bread slices, baking resulted in a significant degradation of scavengers of $\text{ABTS}^{\bullet+}$ radicals regardless of the extraction rate. Moreover, no statistical differences between TAC values of slices and crumbs were observed. The strongest radical-scavenging activities among the samples corresponded to crust. Data indicated that baking degraded some antioxidants in flour, which are naturally occurring or produced during dough preparation, while others are newly generated during formation of the crust. Melanoidins, melanoproteins or prolinated proteins are also known to be formed during baking [17], thus contributing to the antioxidant capacity of rye bread

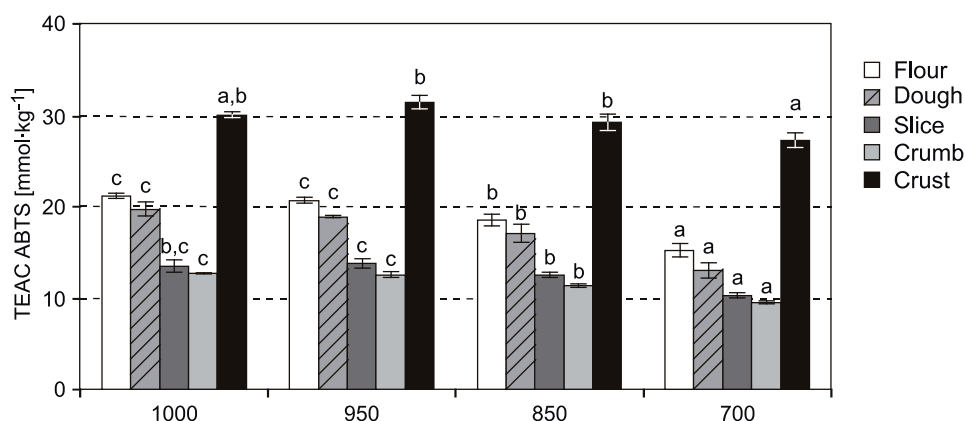


Fig. 3. Total antioxidant capacity of the four types of rye flour, dough and bread.

Vertical bars represent the standard deviation. Data are expressed on a dry matter basis. Different letters within series (flour, dough, slice, crumbs and crust) are statistically different ($P < 0.05$). Extraction rates of flours in horizontal layer are expressed as $\text{g}\cdot\text{kg}^{-1}$.

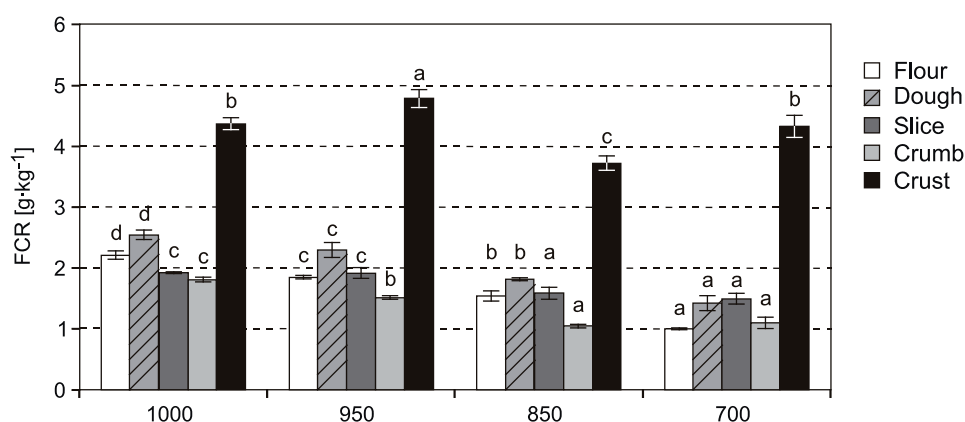


Fig. 4. Total reducing capacity of the four types of rye flour, dough and bread.

Vertical bars represent the standard deviation. Data are expressed on a dry matter basis. Different letters within series (flour, dough, slice, crumbs and crust) are statistically different ($P < 0.05$). Extraction rates of flours in horizontal layer are expressed as $\text{g}\cdot\text{kg}^{-1}$.

Tab. 1. Content of acrylamide (ACR) in rye breads.

Bread type / flour extraction rate	ACR [$\mu\text{g}\cdot\text{kg}^{-1}$]		
	Slice	Crumb	Crust
Type I / 1000	50 ± 1.1^a (31)	16 ± 0.1^b (9)	359 ± 23.0^a (320)
Type II / 950	58 ± 1.4^b (37)	14 ± 0.9^a (8)	464 ± 2.9^b (416)
Type III / 850	62 ± 0.1^c (40)	$15 \pm 0.6^{a,b}$ (9)	367 ± 6.9^a (326)
Type IV / 700	57 ± 2.9^b (31)	$14 \pm 0.1^{a,b}$ (9)	385 ± 12.4^a (343)

Data are expressed as mean \pm standard deviation ($n = 3$) on a dry matter basis. Means in a column followed by different letters are significantly different ($P < 0.05$). Values in brackets express the mean ACR content in fresh portion. Extraction rate is expressed in $\text{g}\cdot\text{kg}^{-1}$.

with a double important function to preserve the quality “sensory and nutritional” of the breads by preventing oxidation of fundamental compounds and to contribute to the intake of compounds possessing antioxidant activity as part of the diet preventing in vivo oxidation processes [35].

Total reducing capacity (TRC)

Data on total reducing capacity of the samples were measured by application of the “quencher approach” in combination with the Folin assay (Fig. 4). Flours samples showed reducing capacity values from $2.21 \text{ g}\cdot\text{kg}^{-1}$ to $1.00 \text{ g}\cdot\text{kg}^{-1}$ and related to the flour extraction rate. Dough preparation slightly increased the reducing capacity due to the releasing compounds able to reduce Folin-Ciocalteu’s reagent (FCR). The highest reducing capacity was recorded in dough from wholemeal flour. This finding was in accordance with that reported by KATINA et al. [34] who found a dependence of antioxidant activity of the fermented dough versus flour extraction rate. Baking resulted in decreased reducing capacity noted in slice and crumb when compared to dough. In contrast, bread crusts were characterized by the highest total reducing capacity, being higher by 56.1, 60.0, 57.2 and 65.5% when compared to the slices prepared from breads formed on flour with extraction rate of 1000, 950, 850 and $700 \text{ g}\cdot\text{kg}^{-1}$, respectively. The total reducing capacity values correlated with PCL-ACW ($r = 0.92$) and with TAC values ($r = 0.92$), thus suggesting that this assay could be applied for characterization of cereal-based products.

Formation of potentially toxic compounds during baking

Acrylamide (ACR) content

As expected, ACR was neither detected in rye flours nor in its respective dough, but ACR was formed during baking as measured in bread slices (Tab. 1). ACR content ranged from $50 \mu\text{g}\cdot\text{kg}^{-1}$ (dry

matter) to $62 \mu\text{g}\cdot\text{kg}^{-1}$ (dry matter) that corresponds to contents ranging from $31 \mu\text{g}\cdot\text{kg}^{-1}$ to $40 \mu\text{g}\cdot\text{kg}^{-1}$ in the fresh slice. These data are in agreement with those previously reported in literature for rye bread [37, 38].

ACR formation did not depend on the extraction rate of the flour. However, the lowest content was in bread slice based on flour with extraction rate of $1000 \text{ g}\cdot\text{kg}^{-1}$ whilst slightly higher, but not statistically different, were in slices from breads based on flour with extraction rates $950\text{--}850 \text{ g}\cdot\text{kg}^{-1}$. This finding was different from data reported by CLAUS et al. [5] for wheat-, rye- and spelt-flour breads. These authors found a positive relationship between ACR content and the extraction rate of the flour used regardless of the type of flour. In that study, protease and amylase activities increased with higher extraction rates, resulting in higher concentrations of asparagine and reducing saccharides in the flour, which are precursors of ACR. In our study, only statistical differences between bread based on flours with extraction rates of $1000 \text{ g}\cdot\text{kg}^{-1}$ and those based on flours with extraction rates of $950\text{--}700 \text{ g}\cdot\text{kg}^{-1}$ was detected. This discrepancy could be attributed to the fermentation step. It is known that fermentation is able to reduce free asparagine in dough and subsequently to reduce ACR formation in bread [37]. In contrast, our results agreed with those reported recently by CAPUANO et al. [39] where ACR content did not exactly parallel the free asparagine content of flour after extraction.

As expected, major formation of ACR was observed in the crust, as it was previously reported [37]. Formation of ACR is basically a surface event in bakery products since the rapid water loss at the surface during baking enhances ACR formation. ACR content in crusts was approximately 27-fold higher than that in the crumb. Therefore, average contribution of ACR formed at crust on the whole slice was estimated as $9.9\% \pm 1.5\%$. Again, no relationship was observed between levels of ACR

in crust or crumb, and the extraction rate of the flour.

According to the European Commission reports, levels of acrylamide in bakery products range from $50 \mu\text{g}\cdot\text{kg}^{-1}$ to $450 \mu\text{g}\cdot\text{kg}^{-1}$ [38]. Daily consumption of acrylamide is 35–40 μg per day, which represents the average exposure for adults (with 70 kg of body weight, bw) close to $0.5 \mu\text{g}\cdot\text{kg}^{-1}$ bw-per day [40]. In Poland, the total consumption of bread is 71 kg per year and person (rye bread 5%, wheat bread 19.2%, and the rest are related to wheat/rye mixed breads) [41]. The monthly intake of rye breads in Poland is on average 0.3 kg per person, with an average ACR content of $34.52 \mu\text{g}\cdot\text{kg}^{-1}$ in fresh slice. Having all this evidence, estimation of daily intake of ACR from fresh rye bread to Polish population was estimated to be $4.93 \text{ ng}\cdot\text{kg}^{-1}$ bw-per day. This value is close to 1% of the average exposure for adults.

HMF and FUR contents

Tab. 2 and Tab. 3 summarize results on the formation of furanic compounds during rye bread making and its respective portions in crumb and crust. HMF contents in bread slices were comparable to those reported in the literature for different bakery products [6, 39]. Average formation of HMF and FUR in fresh bread slices was $31 \text{ mg}\cdot\text{kg}^{-1}$ FW (fresh weight basis) and $0.4 \text{ mg}\cdot\text{kg}^{-1}$ FW, while in crusts was $803 \text{ mg}\cdot\text{kg}^{-1}$ FW and

$18 \text{ mg}\cdot\text{kg}^{-1}$ FW, respectively. Lowest levels of HMF were found in crumbs. HMF contents in crust were approximately 25 fold higher than those determined in fresh bread slices, and 631 fold higher than in crumb. Therefore, average contribution of HMF and FUR from the crust on the whole slices was estimated as $4\% \pm 0.4\%$, and $8\% \pm 2.0\%$, respectively.

Formation of HMF and FUR was evaluated according to the extraction rate applied to flours. The highest levels of HMF and FUR were obtained in bread slice from flour with the lowest extraction rate ($700 \text{ g}\cdot\text{kg}^{-1}$) whilst lower contents, statistically different, were in slices from breads based on flour with extraction rates 1000–850 $\text{g}\cdot\text{kg}^{-1}$. However, HMF and FUR in crust significantly correlated with the flour extraction rate used in the formulation. The finer was the flour used, the higher were FUR and HMF concentrations. Maillard reaction and saccharide degradation are contributing to HMF and FUR formation during bread making. Opposite to ACR, sucrose and hexose degradation contribute the most to HMF formation. Pentose or even HMF degradation at severe baking conditions are responsible for FUR formation. In addition, furanic compounds are also detected in vapours generated during baking and net measurement of furfural in bakery products is a net value between rate of formation and evaporation [42]. Since FUR can be formed

Tab. 2. Content of hydroxymethylfurfural (HMF) in rye breads and its crumb and crust.

Bread type / flour extraction rate	HMF [$\text{mg}\cdot\text{kg}^{-1}$]		
	Slice	Crumb	Crust
Type I / 1000	33.6 ± 4.1^a (21.1)	1.1 ± 0.1^a (0.7)	568.9 ± 12.3^a (507.8)
Type II / 950	44.1 ± 0.3^a (27.7)	0.8 ± 0.1^b (0.5)	701.6 ± 53.0^b (627.7)
Type III / 850	44.6 ± 4.8^a (28.6)	2.2 ± 0.1^c (1.3)	830.3 ± 24.9^c (737.6)
Type IV / 700	87.9 ± 4.8^b (47.9)	1.6 ± 0.1^d (1.0)	1500.9 ± 22.5^d (1339.1)

Data are expressed as mean \pm standard deviation ($n = 3$) on a dry matter basis. Means in a column followed by different letters are significantly different ($P < 0.05$). Values in brackets express the mean HMF content in fresh portion. Extraction rate is expressed in $\text{g}\cdot\text{kg}^{-1}$.

Tab. 3. Content of furfural (FUR) in rye breads and its crumb and crust.

Bread type / flour extraction rate	FUR [$\text{mg}\cdot\text{kg}^{-1}$]		
	Slice	Crumb	Crust
Type I / 1000	1.6 ± 0.3^a (1.0)	0.6 ± 0.001^a (0.4)	14.8 ± 0.5^a (13.2)
Type II / 950	1.6 ± 0.1^a (1.0)	0.7 ± 0.05^b (0.4)	10.7 ± 0.7^b (9.6)
Type III / 850	1.9 ± 0.1^a (1.2)	0.6 ± 0.002^a (0.4)	20.7 ± 0.5^c (18.3)
Type IV / 700	3.3 ± 0.2^b (1.8)	0.7 ± 0.003^b (0.4)	34.1 ± 0.3^d (30.4)

Data are expressed as mean \pm standard deviation ($n = 3$) on a dry matter basis. Means in a column followed by different letters are significantly different ($P < 0.05$). Values in brackets express the mean FUR content in fresh portion. Extraction rate is expressed in $\text{g}\cdot\text{kg}^{-1}$.

after degradation of HMF, its use as an indicator for the development of caramelization process as well as of confirmation of high-temperature manufacturing has been recently suggested [8]. FUR was formed in lower amounts during bread making as compared to HMF, but followed a similar trend. In contrast, presence of heterocyclic compounds like furfurals and furanones in foods are not only linked to safety issues or aroma profile of foods, but also to their antioxidant capacity [35]. Then, there is a dual criterion to estimate the potentially harmful presence of these compounds in bread that should be well addressed.

The estimated daily intake of HMF and FUR from fresh rye bread to Polish population was $4.47 \mu\text{g}\cdot\text{kg}^{-1}$ and $0.18 \mu\text{g}\cdot\text{kg}^{-1}$ bw per day, whilst from crumb of fresh rye bread was $0.12 \mu\text{g}\cdot\text{kg}^{-1}$ and $0.05 \mu\text{g}\cdot\text{kg}^{-1}$ bw per day, respectively. Crust was the major contributor to the exposure, accounting for 97.2% and 69.8% of HMF and FUR, respectively. The estimated daily intake of furfural and precursors of furfural (i.e. furfuryl alcohol and furfuryl esters) from consumption of foods in which they occur naturally, is approximately $300 \mu\text{g}\cdot\text{kg}^{-1}$ bw-per day [43]. Thus, the intake of furfural from rye bread represents only 0.06% of the total intake.

CONCLUSIONS

Rye bread making not only promotes the formation of beneficial substances with antioxidant activity, but also potentially harmful ones. Both processes are concomitant and inherent to the rye bread consumption. The results provided by this study indicate that dough fermentation contributed positively to the generation of lipophilic compounds, exhibiting antioxidant and reducing capacities, which are later diminished during baking. In contrast, baking of rye bread caused a major formation of new water-soluble antioxidants, which are preferably located in the crust, but also ACR, HMF and FUR. Then, any additional mitigation strategy should consider carefully risk and benefits associated to crust. Data provided in the present paper are useful to improve the technology of the rye bread making process on the basis of understanding chemical changes taking place, in order to produce a healthier food.

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REFERENCES

1. Trichopoulou, A. – Soukara, S. – Vasilopoulou, E.: Traditional foods: a science and society perspective. *Trends in Food Science and Technology*, 18, 2007, pp. 420–427.
2. Mondal, A. – Datta, A. K.: Bread baking – a review. *Journal of Food Engineering*, 86, 2008, pp. 465–474.
3. Kroh, L. W.: Caramelisation in food and beverages. *Food Chemistry*, 51, 1994, pp. 373–379.
4. Heiniö, R. L. – Liukkonen, K. H. – Katina, K. – Myllymaki, O. – Poutanen, K.: Milling fractionation of rye produces different sensory profiles of both flour and bread. *Lebensmittel-Wissenschaft und Technologie*, 36, 2003, pp. 577–583.
5. Claus, A. – Schreiter, P. – Weber, A. – Graeff, S. – Hermann, W. – Claupein, W. – Schieber, A. – Carle, R.: Influence of the agronomic factors and extraction rate on acrylamide contents in yeast-leavened breads. *Journal of Agricultural and Food Chemistry*, 54, 2006, pp. 8968–8976.
6. Ramírez-Jiménez, A. – Garcia-Villanova, B. – Guerra-Hernández, E.: Hydroxymethyl-furfural and methylfurfural content of selected bakery products. *Food Research International*, 33, 2000, pp. 833–838.
7. Ait-Ameur, L. – Trystram, G. – Birlouez-Aragon, I.: Accumulation of 5-hydroxymethyl-2-furfural in cookies during baking process: validation of an extraction method. *Food Chemistry*, 98, 2006, pp. 790–796.
8. Rufián-Henares, J. A. – Delgado-Andrade, C. – Morales, F. J.: Analysis of heat-damage indices in breakfast cereals: influence of composition. *Journal of Cereal Science*, 43, 2006, pp. 63–69.
9. Gökmen, V. – Şenyuva, H. Z.: Improved method for the determination of hydroxymethylfurfural in baby foods using liquid chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry*, 54, 2006, pp. 2845–2849.
10. Sadd, P. A. – Hamlet, C. G. – Liang, L.: Effectiveness of methods for reducing acrylamide in bakery products. *Journal of Agricultural and Food Chemistry*, 56, 2008, pp. 6154–6161.
11. Friedman, M. – Levin, C. E.: Review of methods for the reduction of dietary content and toxicity of acrylamide. *Journal of Agricultural and Food Chemistry*, 56, 2008, pp. 6113–6140.
12. Claus, A. – Carle, R. – Schieber, A.: Acrylamide in cereal products: A review. *Journal of Cereal Science*, 47, 2008, pp. 118–133.
13. Mustafa, A. – Fink, M. – Kamal-Eldin, A. – Rosen, J. – Andersson, R. – Aman, P.: Interaction effects of fermentation time and added asparagines and glycine on acrylamide content in yeast-leavened bread. *Food Chemistry*, 112, 2009, pp. 767–774.

14. Zhang, Y. – Zhang, Y.: Study on reduction of acrylamide in fried bread sticks by addition of antioxidant of bamboo leaves and extract of green tea. *Asian-Pacific Journal of Clinical Nutrition*, 16, 2007, pp. 131S–136S.
15. Ciesarová, Z. – Suhaj, M. – Horvathová, J.: Correlation between acrylamide contents and antioxidant capacities of spice extracts in a model potato matrix. *Journal of Food Nutrition and Food Research*, 47, 2008, pp. 1–5.
16. Açar, Ö. Ç. – Gökmen, V.: Investigation of acrylamide formation on bakery products using a crust-like model. *Molecular Nutrition and Food Research*, 53, 2009, pp. 1521–1525.
17. Somoza, V.: Five years of research on health risks and benefits of Maillard reaction products. An update. *Molecular Nutrition and Food Research*, 49, 2005, pp. 663–672.
18. Manzocco, L. – Calligaris, S. – Mastrocola, D. – Nicoli, M. C. – Lerici, C. R.: Review of non-enzymatic browning and antioxidant capacity in processed foods. *Food Science and Technology*, 11, 2001, pp. 340–346.
19. Yoshimura, Y. – Iijima, T. – Watanabe, T. – Nakazawa, H.: Antioxidative effect of Maillard reaction products using glucose-glycine model systems. *Journal of Agricultural and Food Chemistry*, 45, 1997, pp. 4106–4109.
20. Morales, F. J. – Jimenez-Perez, S.: Free radical scavenging capacity of Maillard reaction products as related to colour and fluorescence. *Food Chemistry*, 72, 2001, pp. 119–125.
21. Del Castillo, M. D. – Ferigno, A. – Acampa, I. – Borrelli, R. C. – Olano, A. – Martinez-Rodriguez, A. – Fogliano, V.: In vitro release of angiotensin-converting enzyme inhibitors, peroxy-radical scavengers and antibacterial compounds by enzymatic hydrolysis of glycated gluten. *Journal of Cereal Science*, 45, 2007, pp. 327–334.
22. Michalska, A. – Amigo-Benavent, M. – Zieliński, H. – del Castillo, M. D.: Effect of bread making on formation of Maillard reaction products contributing to the overall antioxidant activity of rye bread. *Journal of Cereal Science*, 48, 2008, pp. 123–132.
23. Bersuder, P. – Hole, M. – Smith, G.: Antioxidants form a heated histidine-glucose model system. Investigation of the copper (II) binding ability. *Journal of the American Oil Chemistry Society*, 78, 2001, pp. 1079–1082.
24. Goya, L. – Delgado-Andrade, C. – Rufián-Henares, J. A. – Bravo, L. – Morales, F. J.: Effect of coffee melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *Molecular Nutrition and Food Research*, 51, 2007, pp. 536–545.
25. Borrelli, R. C. – Mennella, C. – Barba, F. – Russo, M. – Russo, G. L. – Krome, K. – Erbersdobler, H. F. – Faist, V. – Fogliano, V.: Characterization of coloured compounds obtained by enzymatic extraction of bakery products. *Food Chemistry and Toxicology*, 41, 2003, pp. 1367–1374.
26. Morales, F. J. – Martin, S. – Açar, Ö. Ç. – Arribas-Lorenzo, G. – Gökmen, V.: Antioxidant activity of cookies and its relationship with heat-processing contaminants: a risk benefit approach. *European Food Research and Technology*, 228, 2009, pp. 345–354.
27. Rufián-Henares, J. A. – Arribas-Lorenzo, G. – Morales, F. J.: Acrylamide content of selected Spanish foods: survey of biscuits and bread derivatives. *Food Additives and Contaminants*, 24, 2007, pp. 343–350.
28. Rufián-Henares, J. A. – Arribas-Lorenzo, G. – Morales, F. J.: Application of a fast high-performance liquid chromatography method for simultaneous determination of furanic compounds and glucosylisomaltol in breakfast cereals. *Journal of AOAC International*, 89, 2006, pp. 161–165.
29. Popov, I. N. – Lewin, G.: Anti-oxidative homeostasis: Characterisation by means of chemiluminescence technique. *Methods in Enzymology*, 300, 1999, pp. 437–441.
30. Zielińska, D. – Frias, J. – Piskula, M. K. – Kozłowska, H. – Zieliński, H. – Vidal-Valverde, C.: Evaluation of the antioxidant capacity of lupin sprouts produced with presence of selenium. *European Food Research and Technology*, 227, 2008, pp. 1711–1720.
31. Gökmen, V. – Serpen, A. – Fogliano, V.: Direct measurement of the total antioxidant capacity of foods: A ‘QUENCHER’ Approach. *Trends in Food Science and Technology*, 20, 2009, pp. 278–288.
32. Re, R. – Pellegrini, N. – Proteggente, A. – Pannala, A. – Yang, M. – Rice-Evans, C. A.: Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26, 1999, pp. 1231–1237.
33. Liukkonen, K. H. – Katina, K. – Wilhelmsson, A. – Myllymäki, O. – Lampi, A. M. – Kari- luoto, S. – Piironen, V. – Heinonen, S. M. – Nurmi, T. – Adlercreutz, H. – Peltoketo, A. – Pihlava, J. M. – Hietaniemi, V. – Poutanen, K.: Processed-induced changes on bioactive compounds in whole grain rye. *The Proceedings of the Nutrition Society*, 62, 2003, pp. 117–122.
34. Michalska, A. – Ceglińska, A. – Zieliński, H.: Bioactive compounds in rye flours with different extraction rates. *European Food Research and Technology*, 225, 2007, pp. 545–551.
35. Mesa, M. D. – Silvan, J. – Olza, J. – Gil, A. – del Castillo, M. D.: Antioxidant properties of soy protein-fructooligosaccharide glycation systems and its hydrolyzates. *Food Research International*, 41, 2008, pp. 606–615.
36. Katina, K. – Liukkonen, K. H. – Kaukovirta-Norja, A. – Adlercreutz, H. – Heinonen, S. M. – Lampi, A. M. – Pihlava, J. M. – Poutanen, K.: Fermentation-induced changes in the nutritional value of native or germinated rye. *Journal of Cereal Science*, 46, 2007, pp. 348–355.
37. Fredriksson, H. – Tallving, J. – Rosén, J. – Åman, P.: Fermentation reduces free asparagine in dough and acrylamide content in bread. *Cereal Chemistry*, 81, 2004, pp. 650–653.
38. IRMM Database. Monitoring Database. In: European Commission Joint Research Centre,

- Institute of Reference Materials and Measurements [online]. Geel : European Commission Joint Research Centre, Institute of Reference Materials and Measurements, 2008 [cited June 2010]. <<http://www.irmm.jrc.be/html/activities/acrylamide/database.htm>>
39. Capuano, E. – Ferrigno, A. – Acampa, I. – Serpen, A. – Açar, Ö. Ç. – Gökmen, V. – Fogliano, V.: Effect of flour type on Maillard reaction and acrylamide formation during toasting of bread crisp model systems and mitigation strategies. *Food Research International*, 42, 2009, pp. 1295–1302.
40. Dybing, E. – Farmer, P. B. – Andersen, M. – Fennell, T. R. – Lalljie, S. P. – Muller, D. J. – Olin, S. – Petersen, B. J. – Schlatter, J. – Scholz, G. – Scimeca, J. A. – Slimani, N. – Törnqvist, M. – Tuijthelaars, S. – Verger, P.: Human exposure and internal dose assessments of acrylamide in food. *Food Chemical Toxicology*, 43, 2005, pp. 365–410.
41. Piekut, M.: Konsumpcja pieczywa w polskich gospodarstwach domowych. *Przegląd piekarski i cukierniczy*, 7, 2008, pp. 6–18.
42. Ait-Ameur, L. – Rega, B. – Giampaoli, P. – Trystram G. – Birlouez-Aragon, I.: The fate of furfurals and other volatile markers during the baking process of a model cookie. *Food Chemistry*, 111, 2008, pp. 758–763.
43. Bachmann, S. – Meier, M. – Känzig, A.: 5-Hydroxymethyl-2-furfural (HMF) in Lebensmitteln. *Lebensmittelchemie*, 51, 1997, pp. 49–50.

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