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The influence of the starch component on thermal radical generation in flours

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ABSTRACT

Transition metal ions and radicals in flours of various botanical origins with different content of starch have been studied by EPR before and after thermal treatment. The amounts of metal ions, have been determined by ICP OES. Simulations of EPR spectra have revealed the presence of several types of radicals (carbon-centred, tyrosyl and semiquinone) localized in starch and protein fractions of flours. Thermal treatment of flours significantly increased the amount of radicals with a simultaneous decrease of the signal intensity of transition metal ions. The proposed mechanism of thermal generation of stable organic radicals was associated with the redox processes involving transition metal ions, which facilitated the formation of radicals. The dependence between the way starch is treated and the mechanism of radical formation was also shown.

1. Introduction

Flour is the basic source of carbohydrates in the human diet. Cereals and other plants yielding flour are widely cultivated crops all over the world. The biological origin of flour determines its composition, as well as its applicability to bread making. Flours, besides starch being its main fraction, also contain other components, such as mono- and di-carbohydrates, dietary fibre, proteins, fats, vitamins, minerals and water.

During thermal treatment of flours various reactions can occur between their components leading to different properties of the obtained product. Moreover, the process of heating resulting in decomposition of starch and proteins can create free radicals (Yordanov & Mladenova, 2004). The thermal radical formation has been already studied in native and modified starches of different biological origin by Bidzińska, Dyrek, Fortuna, Łabanowska, and Pietrzyk (2004); Łabanowska et al. (2008); Łabanowska, Wesełucha-Birczyńska, Kurdziel, and Puch (2013). It has been found, that radicals are formed on carbon atoms of carbohydrates – amylose and amylopectin, the two major starch constituents. These radicals are stable and may be considered as free electron scavengers, being in this way beneficial for human health. Taking into account more varied composition of flours, radicals created during their thermal treatment could be formed not only on carbohydrate molecules, but also on atoms other than carbon and be localized in other molecules. It can be expected that carbohydrate radicals will be formed mainly in flours containing high amounts of starch, such as those obtained from potato or tapioca, whereas the presence of higher amounts of proteins can facilitate the formation of radicals in peptide structures. The presence of metal ions, especially transition metal ions, can also influence radical generation (Bidzińska et al., 2004; Łabanowska et al., 2008, 2011; Łabanowska, Kurdziel, et al., 2013)

Wheat flour is most commonly used for breadmaking. Recently, in addition to common wheat (*Triticum aestivum ssp. vulgare*) also spelt (*Triticum aestivum ssp. spelta*) has become popular for culinary purposes. Both wheat flours are used for nutritional purposes in two forms: white and wholemeal. The composition of wholemeal flours, containing bran, differs from white flours, so in all processes occurring at high temperatures, one can expect that radicals other than those characteristic of white flours may be created in wholemeal products. It has been already found, that in the case of wholemeal flours higher amounts of radicals are formed during thermal treatment of dough (Andersen, Erichsen, Skibsted, Graversen, & Rodrigues-Filho, 2011). Previous studies were mainly focused on the radical generation processes occurring in flours, as well as in starch upon various treatments, such as high temperatures (Andersen et al., 2011; Bidzińska et al., 2004;

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Yordanov & Mladenova, 2004), UV and γ irradiation (Bertolini, Mestres, Colonna, & Raffi, 2001; Merlin & Fouassier, 1981; Ukai & Shimoyama, 2005; Wasik & Bushuk, 1973), electric discharge (Wasik & Bushuk, 1973), ball milling (Wasik & Bushuk, 1973), ozone treatment (Reichenauer & Goodman, 2003) and by introduction of various additives (Bidzińska et al., 2004; Blennow et al., 2006; Łabanowska et al., 2008, 2011; Paterson, Mitchell, Hill, & Blanshard, 1996; Sriburi, Hill, & Barclay, 1999).

In contrast to starch, in native flours, not subjected to various treatment, small amounts of radical species were found (Reichenauer & Goodman, 2003; Ukai & Shimoyama, 2005). Moreover, the presence of metal ions, especially manganese, was noticed (Reichenauer & Goodman, 2003; Ukai & Shimoyama, 2005); however, no relationship between their amounts and radical creation processes was established.

On the other hand, research on thermal radical generation in native starches and those enriched in transition metal ions (Cu, Fe) showed that metal ions enhanced radical formation with a simultaneous change in metal oxidation states (Łabanowska et al., 2008, 2011; Łabanowska, Kurdziel, et al., 2013).

The aim of this paper was to study the relationship between the process of the thermal generation of radicals and the origin and composition of flours, especially the amount of starch and transition metal ions. The electron paramagnetic resonance (EPR) technique was used to follow the radical formation, particularly their character and number, in flours originating from cereal and tuberous plants. Starch, gluten and bran, which are the basic components of flours, were also considered. Besides radical species, transition metal ions, such as manganese and iron were recorded by EPR. The content of metal ions in flours was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES), whereas the content of starch, soluble sugars, proteins and fats was determined by biochemical analyses.

2. Materials and methods

2.1. Materials and samples description

The samples of flours used in the present study: white and wholemeal wheat, white and wholemeal spelt, corn, potato and tapioca were provided by different local companies. The samples were used without further treatment (native flours) and after thermal treatment.

The appearance of flours depended on their origin and preparation. The wholemeal flours were heterogeneous; the wheat flour was darker and more coarsely ground, whereas the spelt flour was more uniform, lighter and finely ground. The corn and tuber flours were homogenous, uniformly coloured. Bran used for analyses was separated from wholemeal wheat flour by sieving.

2.2. Gluten preparation

Gluten was separated from white wheat flour according to the following method: 100 g of flour was mixed with 50 cm^3 of distilled water. The obtained dough was immersed into 250 cm^3 of water for 5 min and washed out in the stream of running water. Then gluten was air-dried at room temperature and ground.

2.3. Determination of biochemical components of flours

In order to determine the content of soluble sugars and starch, 1 g of flour was extracted using 80% (v:v) ethanol and the supernatant was used for soluble sugar determination, whereas the pellets were used for starch determination. The pellets were hydrolyzed with 35% $HClO_4$ and the supernatant was taken for further analysis. Sugar and starch content was estimated spectrophotometrically (λ = 625 nm, Ultrospec II 4051 LKB Biochrom, UK) using the anthrone method (Janeczko et al., 2010).

Fats were extracted from 1 g of flour with 10 cm^3 of mixture of chloroform:methanol (2:1 v/v). The supernatant phase was shaken with 30 cm^3 of a 1% solution of KCl to separate the chloroform phase containing fats. The chloroform layer was then evaporated to dryness under N_2 and the residue was weighted.

The content of soluble proteins was analyzed according to the Bradford (1976) method. Flour (50 mg) was mixed with 5 cm^3 of distilled water and 10 μl (10^{-2} cm^3) of the obtained supernatant phase was mixed with the Bradford reagent. After 20 min of incubation at room temperature, absorbance (λ = 595 nm) was measured using a Lambda Bio 20 (Perkin-Elmer, Germany) spectrophotometer. Bovine serum albumin (Sigma–Aldrich) was used as the calibration standard.

2.4. Determination of metal ions content

Samples were digested with concentrated nitric acid (Suprapure Merck, Darmstadt, Germany) in a microwave assisted system (Anton Paar MULTIWAVE 3000, Austria) at 513 K and 60 bar (max). Digests buffered to 1 g/dm^3 with lithium nitrate were analyzed directly (Ca, Cu, Fe, Mn, Na) and after 10-times dilution (K and Mg) with ICP atomic emission spectrometer, Perkin Elmer Optima 2100, USA, at following wavelengths: Ca – 317.933 nm; Cu – 327.393 nm; Fe – 238.204 nm; Mn – 257.610 nm; Na – 589.592 nm; K – 766.490 nm and Mg – 285.213 nm, respectively.

Standards from Merck, Darmstadt, Germany (ICP multi-element standard solution IV) and Perkin Elmer, Shelton, USA (multi-element calibration standard No. 3), treated in the same way as the samples, were used for calibration.

2.5. Thermal treatment of the samples

The samples of investigated flours (about 100 mg) were placed in quartz EPR tubes and heated in an oven for 30 min at 423 K, followed by 30 min at 483 K. Prior to EPR measurements the tubes with the samples were cooled to room temperature and closed with a paraffin membrane.

2.6. Ozonation

The samples of wheat starch and flour were treated with ozone for 30 min, using an FM-300 ozonator (Grecos, Poland).

2.7. UV irradiation

The samples of wheat and potato starches and flours were placed in quartz EPR tubes and irradiated with a UV Irradiation System ER 203UV (Bruker, Karlsruhe, Germany) in situ in an EPR resonator (Elexsys Super High Sensitivity Probehead E41 22011).

2.8. EPR measurements

EPR measurements were performed with an X-band Bruker ELEXSYS 500 spectrometer (Karlsruhe, Germany) with 100 kHz field modulation. The spectra were recorded at room temperature and 77 K with modulation amplitudes of 0.5 mT and 0.1 mT and microwave powers 5.0 mW and 3.0 mW. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used as a *g*-factor standard. The integral intensity of radical signals was related to the same mass of the samples and was compared with the integral intensity of the signal of DPPH with the known number of spins (1.12×10^{15} spins/g). EPR parameters: g factor value, hyperfine splitting constant *A*, peak-to-peak line width ΔB_{pp} were found by the simulation procedure

Table 1
The biochemical components of flours.

Flour	Starch (%)	Soluble sugars (%)	Fat (%)	Soluble proteins (%)
White wheat	56.31 ± 3.4	1.52 ± 0.01	0.37 ± 0.14	11.54 ± 0.17
Wholemeal wheat	65.96 ± 1.3	2.60 ± 0.04	1.66 ± 0.22	12.63 ± 0.53
White spelt	43.52 ± 3.3	2.01 ± 0.04	2.41 ± 0.14	11.39 ± 0.04
Wholemeal spelt	50.34 ± 3.2	2.02 ± 0.10	1.72 ± 0.24	13.46 ± 0.43
Corn	48.27 ± 4.9	1.50 ± 0.14	4.52 ± 0.29	7.55 ± 0.31
Potato	85.34 ± 4.1	0	0.68 ± 0.15	5.84 ± 0.48
Tapioca	81.01 ± 3.9	1.26 ± 0.06	0.57 ± 0.39	6.52 ± 0.79

using the modified programme SIM 32 (Spałek, Pietrzyk, & Sojka, 2005). The theoretical spectrum was the sum of all individual signals, with appropriate contributions, originating from the assumed models of paramagnetic centres.

2.9. Raman spectra

The Raman spectra of potato and wheat starch, as well as the respective flours and wheat bran were recorded with an inVia Raman microspectrometer produced by Renishaw (UK), working in confocal mode. Samples of wheat flour, starch and bran, as well as potato flour and starch were excited with a 785 nm laser line of HP NIR diode laser Renishaw (UK).

3. Statistical analysis

Biochemical analyses were repeated three times and averaged. The standard deviations (±SD) are given in Table 1. The analyses of metal content were also repeated three times. The relative standard deviations (RSD) for determination of particular metals content are given in Table 2. The quantitative calculations of radical amounts were based on data obtained from three spectra measured for each of two samples. The error bars (±SD) are marked in Fig. 2. The calculations were performed using programme Statistica 9 (Statsoft Inc., USA). The maximum error of the quantitative measurements (5 repetitions) calculated for DPPH standard was 0.05×10^{15} spins/g.

The accuracy of EPR parameters was ±0.0002 for g values and ±0.05 mT for parameters of hyperfine splitting constant A. The accuracy of the estimation of metal ion signal parameters from the spectra was ±0.1 for g values and ±0.5 mT for parameters A.

The recording of Raman spectra was repeated five times for each sample.

4. Results and discussion

The chemical composition of the investigated flours is presented in Table 1. Potato and tapioca flours exhibited the highest content of starch, whereas flours originating from cereal plants, especially wholemeal wheat and spelt flours were richer in proteins. From among the studied flours, corn meal had the highest content of fat. The metal contents of flours also depended on their botanical origin (Table 2). Potato flour showed the highest content of sodium and calcium, whereas the amounts of other elements were higher in the remaining meals, particularly in the wholemeal flours containing bran. It was found by Butt, Qamar, Anjum, Aziz, and Randhawa (2004) that the aleurone cells layer, which adheres to bran, contained: Fe (0.074–0.103 mg/g), Cu (0.008–0.016 mg/g), Mn (0.072–0.144 mg/g), which accounted for about 60% of their content in the wheat grains. The comparison of the contents of Fe, Cu and Mn showed their much higher level in wholemeal wheat flours than in white meals, indicating that these metals were concentrated mainly in bran (Table 2). From among white wheat flours, spelt flour showed the higher content of minerals, with exception of calcium. On the other hand, the corn flour exhibited the lowest content of micro and macro elements in the group of cereal wholemeal flours.

The presence of some microelements in flours was confirmed by electron paramagnetic resonance studies which provided additional information of their bonding to flour components. Fig. 1 presents the EPR spectra of wholemeal and white wheat flours, as well as bran recorded at room temperature and at 77 K. The main signal of these spectra consisted of six equally spaced hyperfine lines with $g = 2.0$ and $A = 8.7$ mT, overlapped on the broad signal ($\Delta B_{pp} \cong 40$ mT) also with $g = 2.0$. This line may result from high spin manganese ions, coupled by dipole–dipole interactions, present in protein structures (Miller & Brudvig, 1991). HFS lines were characteristic of manganese ions in surroundings of oxygen atoms of the $[Mn(H_2O)_6]^{2+}$ complex and were very often observed in many kinds of biological materials (Łabanowska, Filek, Kurdziel, et al., 2012; Lisowski, Jezierski, & Bylińska, 1993; Miller & Brudvig, 1991). Between the allowed lines of HFS the forbidden ones were visible. Analysis of the intensity ratio of the forbidden to allowed lines of HFS in the investigated spectra (Fig. 1, insert) provided additional confirmation of manganese signals' attribution. This ratio calculated for the HFS line responsible for transition $m_I = 3/2$ was equal to 0.37 for wholemeal wheat flour, as well as for bran and 0.53 for white wheat flour. It was much higher than 0.13, the value obtained from the spectrum of MnO/MgO (Łabanowska, 1993), indicating a higher value of parameter D (the value of zero-field splitting for high spin Mn(II)), which was characteristic of manganese–protein complexes (Stich et al., 2007). The narrow signal R with g of about 2.0, situated between the third and fourth line of HFS manganese structure, was characteristic of radical species and would be

Table 2
The content of metals in flours calculated from ICP atomic emission spectra.

Flour	Metal content (mg/g)						
	Na	K	Mg	Ca	Cu	Fe	Mn
White wheat	0.005	1.075	0.184	0.213	0.001	0.008	0.004
Wholemeal wheat	0.007	3.867	1.468	0.326	0.004	0.030	0.041
White spelt	0.006	1.539	0.401	0.163	0.002	0.011	0.006
Wholemeal spelt	0.010	2.835	0.846	0.215	0.004	0.022	0.017
Corn	0.001	1.913	0.464	0.024	0.001	0.011	0.002
Potato	0.029	0.225	0.123	0.464	0.000	0.002	0.000
Tapioca	0.005	0.192	0.029	0.223	0.000	0.018	0.001
RSD (%)	0.3–1.4	1.8–5.1	0.3–2.9	0.4–2.5	0.1–0.5	0.6–1.8	0.1–1.1

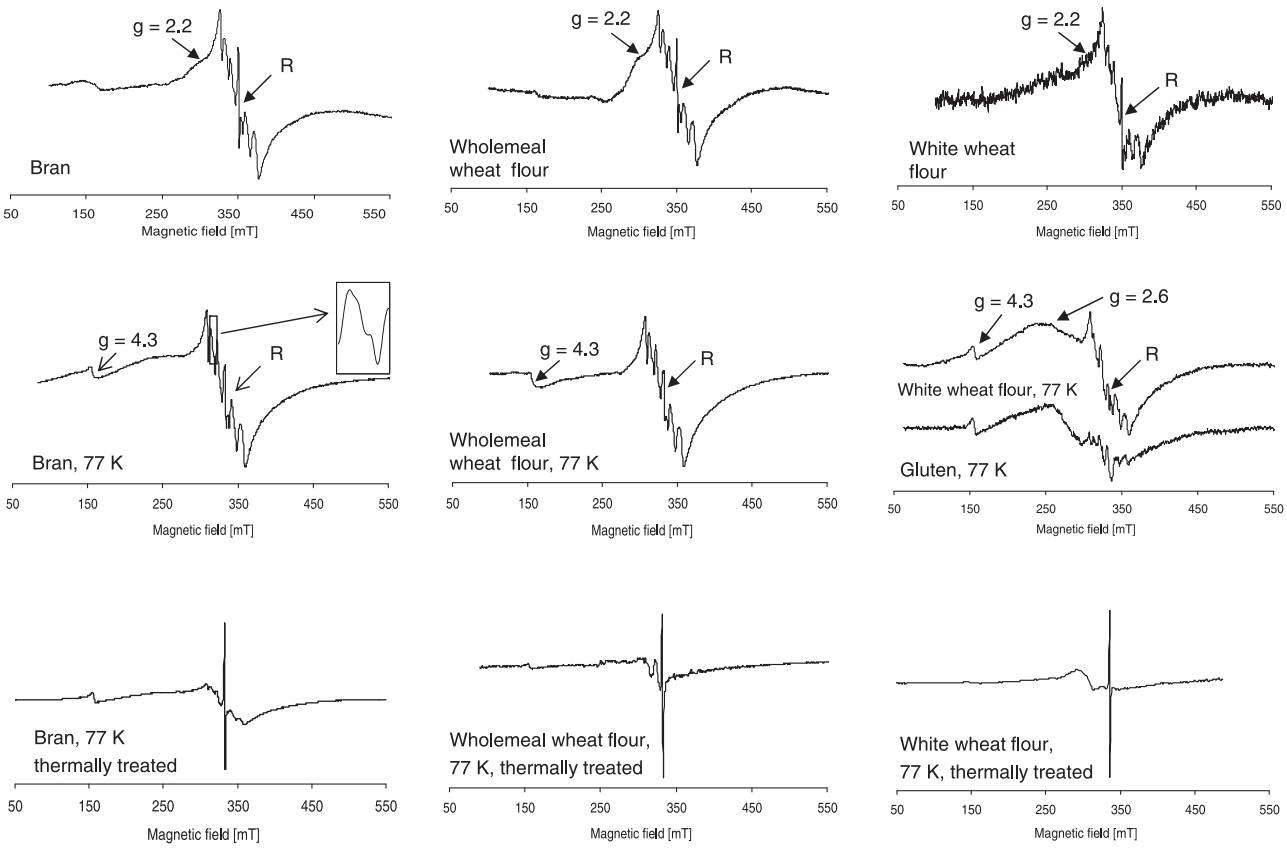

Fig. 1. The experimental spectra of flours, gluten and bran, before thermal treatment, recorded at room temperature and at 77 K and thermally treated, recorded at 77 K, in the range of 500 mT.

discussed later. The signal of Mn(II) recorded at room temperature was the weakest in white flour, whereas it was the most intensive in the spectrum of bran (about fifteen times higher), which was consistent with the results of ICP atomic emission analysis (Table 2). The intensity of the radical signal was also higher in the spectrum of bran. The shoulder at $g \cong 2.2$ visible in all spectra recorded at room temperature was more intensive in the spectra of wholemeal flours and bran than in that of white wheat flour. In the low field part of the spectra the signal with low intensity at $g \cong 4.3$ could also be seen. Lowering the registration temperature to 77 K led to an increase in the intensity of this signal with simultaneous manifestation of its anisotropy. This signal could be attributed to non-haem Fe(III) high spin species with rhombic symmetry and was often observed in many biological systems (Baader, Bill, Trautwein, Bruchelt, & Matzanke, 1996; Cheesman et al., 1992; Miller & Brudvig, 1991). At low temperature the signal at $g \cong 2.2$ disappeared from the spectra, which was a characteristic feature of antiferromagnetically coupled paramagnetic species (Baader et al., 1996; Łabanowska, Kurdziel, et al., 2013; Włoch, Sulikowski, Dula, & Serwicka, 1996). The behaviour of this signal could suggest that paramagnetic centres derived from the clustered, magnetically ordered iron centres and could originate from Fe—O—Fe clusters, ferric oxides, oxyhydroxides and/or phosphates (Sutter, Wasowicz, Howard, Hossner, & Ming, 2002). Such inorganic centres can be found in plant tissues, in which they were probably accumulated in the "iron-core" of ferritin, an iron storage protein (Baader et al., 1996), also present in wheat grains (Zielińska-Dawidziak, Gawlak, & Warchalewski, 2007). Simultaneously, with the disappearance of the signal at $g \cong 2.2$, a new broad line at $g \cong 2.4$ became visible. It was well separated in the spectrum of white wheat flour, but in the spectra of bran and wholemeal flours only a rising of the zero line was observed. The broad line at $g \cong 2.4$ exhibited the behaviour consistent with Curie–Weiss law, suggesting its origin from non coupled iron centres. The same line appeared also in the spectrum of ferritin recorded at 73 K by Baader et al. (1996). Similarly, as it was observed in our spectrum, its appearance was accompanied by vanishing of the line at $g \cong 2.2$. In order to explain the source of the signal at $g \cong 2.4$, the spectrum of gluten, the protein fraction of flour, rich in iron ions (0.024 mg/g), was recorded (Fig. 1). At 77 K this spectrum was similar to that of white wheat flour and it also exhibited the signal at $g \cong 2.4$; however, the intensities of manganese HF lines and radical signal, overlapping the fourth line of manganese hyperfine structure, were lower than in wheat flour spectrum. On this basis, we ascribed tentatively the broad signal in the white wheat flour to Fe(III) species bonded to proteins. Taking into account the behaviour of these two iron centres (with $g \cong 2.2$ and $g \cong 2.4$), we suggest that they originate from ferritin. Besides inorganic core-iron, the ferritin protein shell comprises other ferric and ferrous ions, located in ferroxidase centres, where iron oxidation occurs (Bertini et al., 2012; Pfaffen, Abdulqadir, Le Brun, & Murphy, 2013). As it was suggested by Bou-Abdallah (2010), the oxidation of Fe(II) to Fe(III) by O_2, giving Fe(III)–O–Fe(III), was followed by the formation of protein radical involving the tyrosine residue. Oxygen-bridged iron species, being probably antiferromagnetically coupled, could be observed in EPR spectra rather at room temperature, therefore the signal at $g \cong 2.4$ (observed at 77 K) might not originate from them. Moreover, the studies of Masuda, Goto, Yoshihara, and Mikami (2010) revealed the presence of an additional centre in plant ferritin, in which non-coupled iron ions were surrounded by six oxygen atoms, one from tyrosine, one from glutamic acid and four from water molecules. These centres could be a source of such signal.

The intensities of the remaining signals, visible in EPR spectra of wheat flour and bran, attributed to a radical and manganese complex increased upon decreasing temperature. The intensity of the radical signal was about two times higher, whereas that of manganese lines was about four times higher.

Spectra of spelt (wholemeal and white) flours were very similar to those of wheat, whereas analogous spectra observed for flours obtained from tuberous plants and corn had much lower intensities. The signal at $g = 4.3$ and a broad line at $g = 2.4$ (observed at 77 K) were more intensive in the spectra of corn and tapioca flours than in potato one containing a smaller amount of proteins. Radical signals were weaker in the spectra of flours from tuberous plants and white wheat flour than those observed in other cereal meals.

After thermal treatment of the samples, the spectra of flours and bran significantly changed (Fig. 1). In the spectra recorded at room temperature and 77 K, the signal of the radical became the main line, whereas the signals attributed to metal ions strongly decreased their intensities. The decrease of the intensities of metal ions signals indicated that new metal species, non-active in EPR, were formed. They could be non-Kramer ions d^4Mn(III)/Fe(IV) or d^6Mn(I)/Fe(II) not visible in EPR spectra under the conditions used. The strong increase of dipole-dipole interaction, resulting from thermal degradation of metal-aqua/protein complexes, leading to the vanishing of EPR signals also could not be excluded.

Analysis of radical signals present in the spectra of investigated samples before and after thermal treatment was performed for spectra recorded at room temperature and at 77 K in the range of 5 mT. In native samples of flours the number of spins calculated from the spectra recorded at room temperature was rather low (Fig. 2). The highest amounts of radicals were found in wholemeal flours, which was caused by the presence of bran, rich in radical species (Fig. 2). Flours from tubers and white wheat flour showed very weak signals. Simulation of the spectra of radicals revealed the presence of two signals (I and II) (Fig. 3). The parameters of these signals for wholemeal flours and white spelt were practically the same, however, with different contributions to the spectra. The comparison of these signals with those found in the spectrum of bran showed their similar EPR characteristics and hence we assumed the same origin of these signals. The isotropic signal with $g = 2.0048$ (signal I) was typical of a stable semiquinone radical (Eriksson, Himo, Siegbahn, & Babcock, 1997; Leprince, Atherton, Deltour, & Hendry, 1994; Polat & Korkmaz, 2008). Such species could be created as a result of disturbed balance between quinone-semiquinone-hydroquinone occurring for example upon drought stress when reactive oxygen species (ROS) were formed. Semiquinone radicals have often been observed in plant material, subjected to the drying process (Łabanowska, Filek, Kościelniak, et al., 2012; Leprince et al., 1994). Indeed, the highest contribution of this signal to the integral intensity of the spectrum (about 80%) was observed for bran and bran-containing wholemeal flours, whereas in white spelt flour it reached only 20%. The presence of semiquinone radicals in wheat grains was suggested by Reichenauer and Goodman (2003), however, the existence of phenoxyl radical species were also taken into account. The second signal with $g = 2.0056$ (signal II) could be simulated as a doublet with $A = 0.7$ mT. Its splitting probably resulted from the interaction between the magnetic moment of an unpaired electron and nuclear spin $I = 1/2$, for example ^{1}H. In the case of tuber and corn flours also two signals were used for simulation, but no HFS was observed. Signal I was similar to that observed in wholemeal flours spectra, whereas instead of signal II, the isotropic line (signal III) with $g = 2.0062–2.0068$ appeared. Both signals II and III exhibited g values in the range of those characteristic of carbon-centred radicals in carbohydrates (Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013). In order to confirm that ROS created under stress conditions were responsible for the formation of semiquinone species in bran, this fraction of meal was subjected to ozone treatment. After such treatment the character of the EPR signal did not change, but its intensity increased two times, suggesting the same nature of radical created in bran before and after ozonation. The increase of radical signal intensity in the spectra of grains originating from wheat plants stressed by ozone, compared with control samples was observed by Reichenauer and Goodman (2003). The ozone treatment was also performed on white wheat flour and wheat starch. The native sample of starch did not exhibit EPR signals, whereas those of untreated white flour were very weak. After being exposed to ozone, in both cases new signals appeared (Fig. 4), different from those observed in bran, suggesting that in these samples the action of ROS generated radicals of different nature. Simulation revealed that starch spectrum consisted of two signals: a single line O-1 with $g = 2.0049$ and a doublet O-2 with $g = 2.0044$ and $A = 0.6$ mT. These parameters were similar to those observed for the signals in the spectra of thermally treated starch enriched with Cu(II) or Fe(III). Those signals were assigned to the radicals formed by abstraction of hydrogen atoms from C(5) atoms of glucose units of starch by intermediate alkoxy radicals generated in the presence of transition metal ions at the C(6) atom of the glucose unit (Łabanowska et al., 2008, 2011; Łabanowska, Kurdziel, et al., 2013). In the case of wheat flour similar main signals appeared after ozonation, but additionally small intensive side lines were visible (Fig. 4). They were simulated as a signal with $g = 2.0028$ and $A_1 = 0.8$ mT, $A_2 = 3.6$ mT, which could be ascribed to carbon centred radicals localized in molecules of soluble carbohydrates (Łabanowska, Filek, Kurdziel, et al., 2012; Łabanowska, Filek, Kościelniak, et al., 2012; Yamauchi, Sugito, & Kuzuya, 1999) or in proteins (Aydin, Baskan, & Osmanoglu, 2009), present in flours. Signals O-1 and O-2 were also generated in starch, as well as in flours (wheat and potato) subjected to UV irradiation in the presence of air (Fig. 4). In such conditions not only was the UV energy delivered to the samples, but also water molecules contained in the air and in starch or flour samples could react with ozone formed upon UV irradiation from oxygen ($O_2 \xrightarrow{UV} 2O$; $O + O_2 \rightarrow O_3$), to produce reactive oxygen species ($HO^{\bullet}$, $HO_2^{\bullet}$). These radicals could abstract hydrogen from carbohydrate molecules leading to carbon centred radicals, similar to those created upon ozonation. The character of these radicals was different from those created in thermally treated native starch. In the latter case the formation of radicals occurred via breaking of glycosidic bonds and an unpaired electron was located at the carbon atom C(1) and/or C(6), near the broken bonds, whereas reactive oxygen species with small molecular weight could penetrate into starch molecules and detach hydrogen from other carbon atoms. Moreover, the binding energy of the C—H bond is higher than that of the glycosidic bond, so the breaking of the former requires higher energy (UV or γ) (Bertolini et al., 2001), whereas thermal or even mechanical energy is sufficient to break the glycosidic bond (Kuzuya, Yamauchi, & Kondo, 1999). Hence, it can be suggested that the mechanism of radical formation leading to gradual degradation of starch depends on the way of disturbing its structure.

At 77 K the shape of radical signals became more complex than that observed at room temperature (Fig. 3) and the intensities of spectra of wholemeal wheat and corn flours, as well as bran, increased about four times in comparison with the signals measured at room temperature, whereas the spectra of remaining flours, white wheat and tuber, exhibited a weaker increase. It suggested that the species responsible for signals in wholemeal wheat and corn flours spectra were situated mainly in cereal bran. Bran, containing high amounts of phenolic-like compounds could be the source of stable phenoxyl or semiquinone radicals (Leprince et al.,

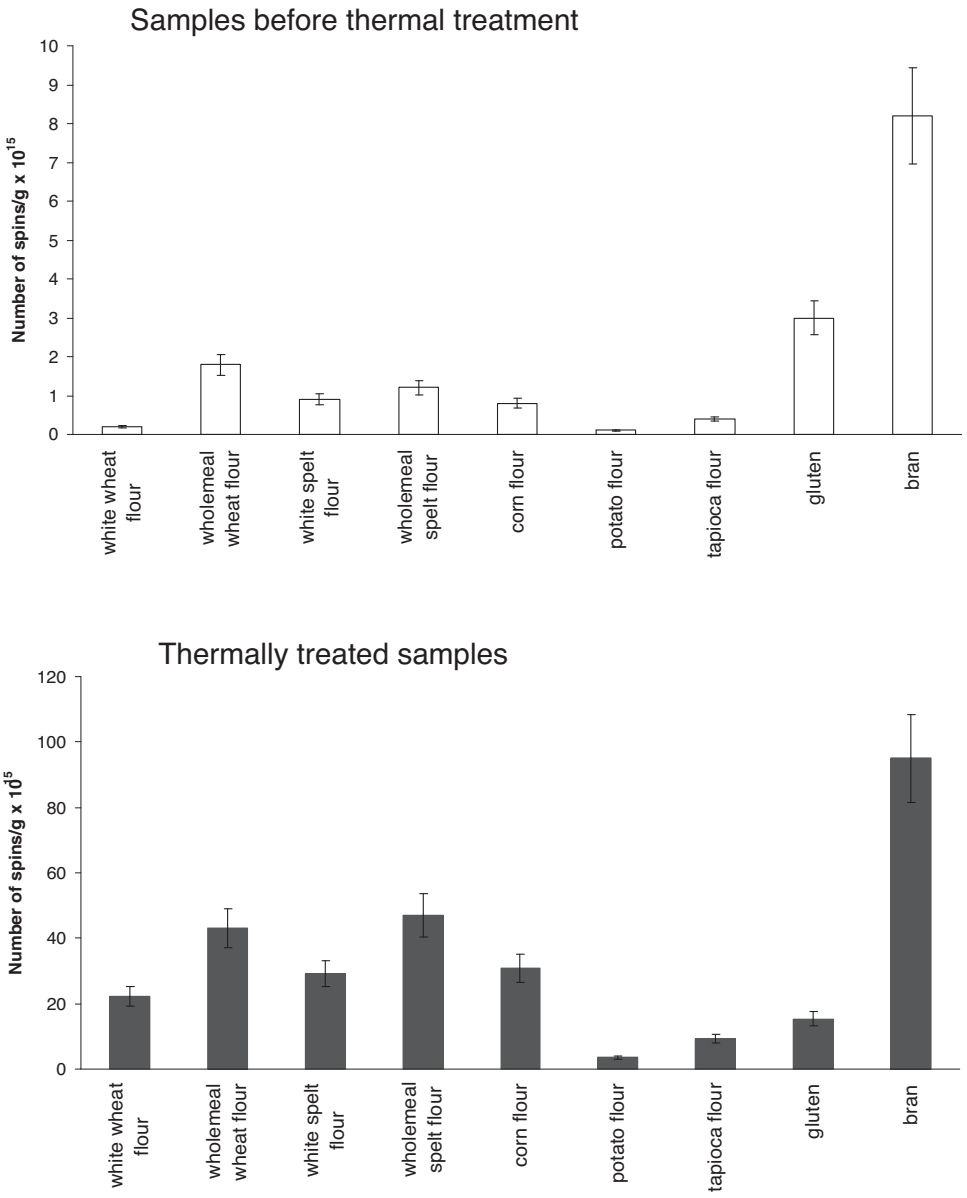

Fig. 2. The number of radicals found in flours, gluten and bran before and after thermal treatment.

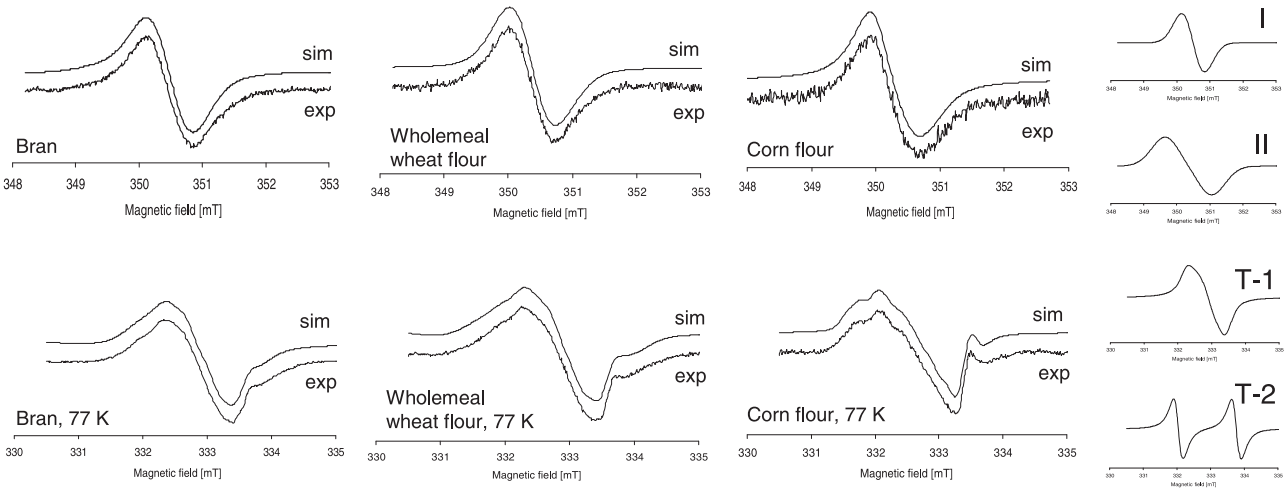

Fig. 3. The experimental and simulated spectra of flours and bran, before thermal treatment, recorded at room temperature and at 77 K in the range of 5 mT. I and II – particular signals used to simulate EPR spectra recorded at room temperature, T-1, T-2 – particular signals used to simulate EPR spectra recorded at 77 K.

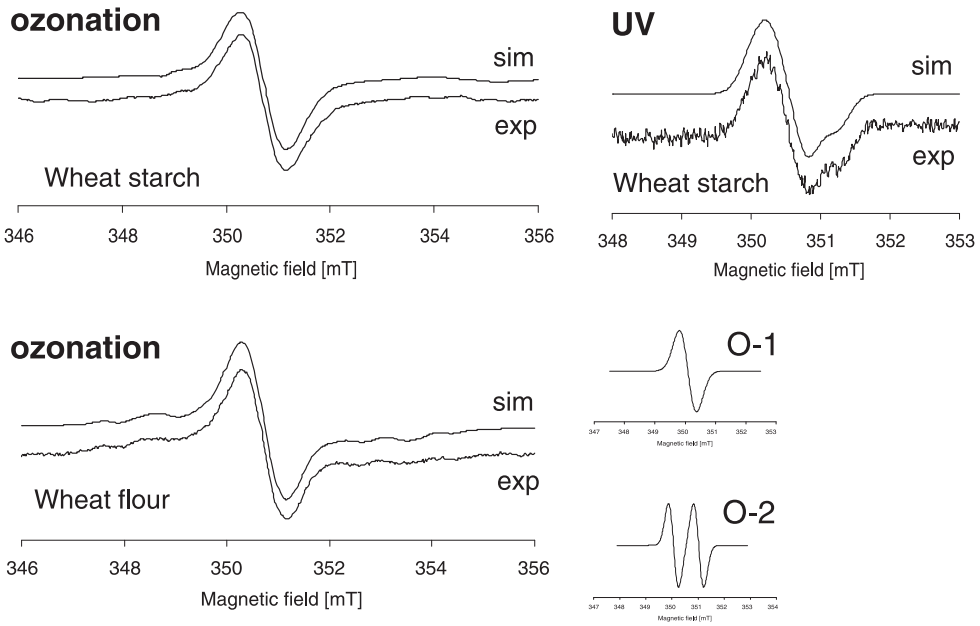

Fig. 4. The experimental and simulated spectra of wheat starch and flour subjected to ozone treatment and the spectrum of UV irradiated wheat starch recorded at room temperature. O-1, O-2 – particular signals used to simulate EPR spectra.

1994; Yamaji, Saiful, Baba, Yamauchi, & Yamauchi, 2007). Simultaneously, the aleurone layer adhering to bran, rich in protein compounds, could supply radical species of other types localized in the protein matrix. The shapes of the spectra of wholemeal wheat and corn flours, as well as bran, were similar; however, the intensity of corn spectrum was lower and signal was better resolved (Fig. 3). The simulation procedure revealed that the anisotropic signal with $g_1 = 2.0083$, $g_2 = 2.0047$, $g_3 = 2.0015$ ($g_{av} = 2.0048$) was the main signal (with contribution to the spectrum accounting for about 80%) (signal T-1) of the spectra of wholemeal wheat and spelt flours and bran. These values are characteristic of the tyrosyl radical (Hofbauer et al., 2001; Miller & Brudvig, 1991; Schünemann et al., 2004; Tomter et al., 2012). Hence, we initially assumed that the observed signal originated from the tyrosyl radical in which an unpaired electron was delocalized on the phenoxyl ring of the tyrosine residue. Good fitting of theoretical spectra to experimental ones required the addition of the second signal (signal T-2) with g value equal to g_{av} of the main signal and $A^{H\beta} = 1.8$ mT. Such splitting was observed for the tyrosyl radical and was attributed to the spin coupling between the unpaired electron and one of the hydrogen atoms situated at C_β atom of the tyrosine molecule (Schünemann et al., 2004; Tomter et al., 2012). Due to the limited interaction with the second H_β atom (Tomter et al., 2012), it was not possible to observe this splitting in the X-band. In the spectrum of corn flour the values: $g_1 = 2.0090$, $g_2 = 2.0045$, $g_3 = 2.0013$ ($g_{av} = 2.0049$) were found for the main signal (T-1), but in this case the interaction of the magnetic moment of the unpaired electron with the spins of ring protons should be additionally taken into account. These interactions were rather weak ($A_1^H = 0.3$ mT, $A_2^H = 0.3$ mT, $A_3^H = 0.1$ mT) and they were revealed because of the decrease in interactions between radical species caused by smaller concentration of radicals in corn flour in comparison with bran and wholemeal flour. The split connected with interaction with H_β atom (signal T-2) was similar to that found for wholemeal flour and bran signals and was equal to 1.9 mT. The relatively high value of g_1 found in our spectra could indicate that the spin density was shifted towards the oxygen atom (Schünemann et al., 2004), suggesting a lack of interactions with the hydrogen atom of the hydroxyl group. The good agreement of g-tensor components values, as well as the value of $A^{H\beta}$ of signals recorded in flours and bran with those obtained from experimental and theoretical studies on model systems (Barry, El-Deeb, Sandusky, & Babcock, 1990; Himo, Gräslund, & Eriksson, 1997; Hofbauer et al., 2001; Schünemann et al., 2004; Tomter et al., 2012) confirmed our attribution of the signal to the tyrosyl radical. The signal with similar shape, as observed by us, was found after γ-irradiation of durum wheat by Korkmaz and Polat (2000); however, its interpretation was different than our approach. The stable tyrosyl radical participates in redox processes in different biological systems, such as PSII and various enzymes and its action is connected with the proximity of transition metal ions, active in electron transfer (Himo et al., 1997; Schünemann et al., 2004; Tomter et al., 2012). In plants, a stable tyrosyl radical is formed naturally during life processes and, as was mentioned earlier, its formation, for example in ferritin, was connected with the iron oxidation process (Bou-Abdallah, 2010). On the other hand, the formation of the tyrosyl radical during processes connected with flour production, which include grain drying, milling etc., should also be considered. The loss of water during maturation and drying of grains influenced mostly the seed coats. Indeed, the most intensive signals were observed in bran. Desiccation could injure protein structures, leading to strong disturbances, also in tyrosine residues. The very intensive tyrosyl radical signals were observed in bran containing samples with high transition metal ions content, which may suggest that radical formation was influenced by the presence of these ions. After ozonation the intensities of tyrosyl, as well as semiquinone signals in bran increased about two times. Also the very weak signal, observed at room temperature and at 77 K, in white flour showed higher intensity.

Although the g_{av} of the tyrosyl radical signal recorded at 77 K was similar to that found for semiquinone signal (signal I) observed at room temperature in the spectra of flours and bran, we claimed, that both signals originated from two different centres: the tyrosyl radical well visible at 77 K and the semiquinone radical giving an isotropic single line at room temperature. At 77 K the semiquinone signal probably became resolved into several hyperfine lines of much smaller intensities and with small A values, which were covered by a more intensive tyrosyl radical signal. The recorded Raman spectra of starch, flour and bran allowed the structures in which radical formation occurred to be characterized (Fig. 5a) The spectra of starches, wheat and potato, as well as those of potato

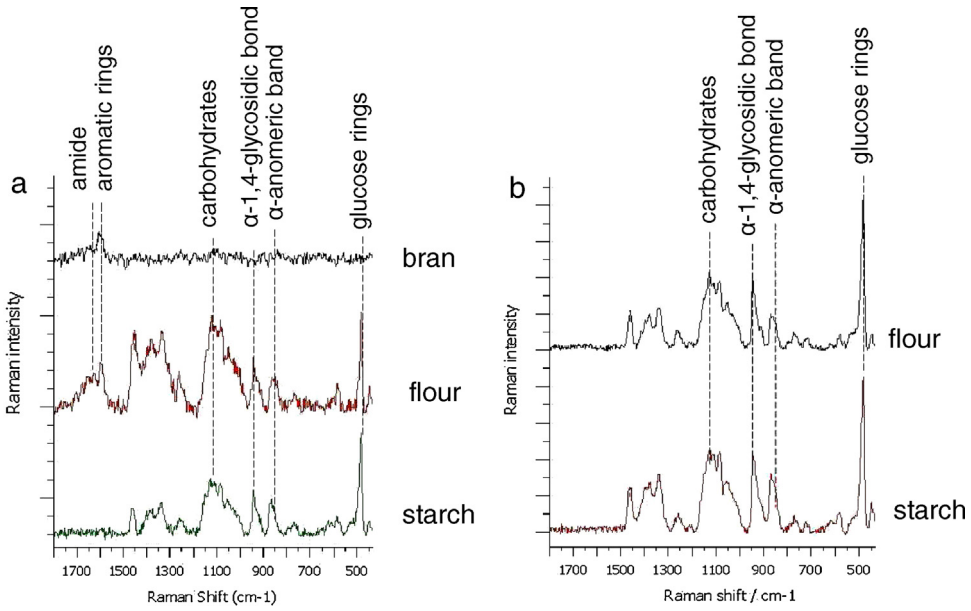

Fig. 5. Raman spectra of wheat bran, flour and starch (a) and potato flour and starch (b) recorded in the range 450–1800 cm^{-1}.

flour (Fig. 5a and b) exhibited bands characteristic of the glucose molecule, the unit building starch chains (Cael, Koenig, & Blackwell, 1973; Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013; Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Sepioło, 2013). The band at 480 cm^{-1} was typical of the starch skeleton, whereas a peak known as the α-anomeric band of carbohydrates appeared at 850 cm^{-1}. The band at about 940 cm^{-1} was due to skeletal mode vibrations of the α-1,4 glycosidic linkage (C—O—C) in starch. The group of bands at ca. 1100 cm^{-1} originated from the carbohydrate C—O stretch (Cael et al., 1973; Kizil, Irudayaraj, & Seetharaman, 2002; Sekkal, Dincq, Legrand, & Huvenne, 1995). Hence, in both types of starch and flour, the formation of similar radicals of carbohydrate character could be expected.

In the spectrum of wheat flour (Fig. 5a), containing higher amount of proteins, additional bands appeared. One of them – a broad band in the range of 1650–1670 cm^{-1} (C=O stretching and N—H wagging vibrations) could originate from amide bonds, whereas the band at 1580 cm^{-1} was characteristic of ν(C=C) aromatic ring chain vibrations (Colthup, Daly, & Wiberley, 1990). The same band (1580 cm^{-1}) was also visible in the spectrum of bran; however, its intensity was smaller than in the spectrum of flour. The lack of the amide band in the Raman spectrum of bran could result from the fact, that only the outer side of bran was analyzed. It meant that the outer layers contained mainly aromatic compounds, for instance phenols. Hence, we proposed that semiquinone radicals could be created in bran in the process of the oxidation of phenols, whereas proteins contained in flours and the aleurone layer were the source of tyrosyl radicals. Carbon-centred radicals would be formed in starch, the main component of flour.

After thermal treatment an increase in the signals intensities occurred in the spectra of all flours and bran taken at room temperature and at 77 K. The highest growth of the signals was observed for bran and wholemeal flours, whereas in the case of tuber flours this increase was much lower (Fig. 2). The spectra of bran and wholemeal wheat and spelt flours recorded at room temperature were symmetric and structureless, whereas those of tuber meals and white wheat flour exhibited a more complex shape (Fig. 6). Simulation of the former was performed using two signals with g factors equal 2.0041 (signal X) and $g = 2.0045$ (signal Y). The signal with a higher g value was broader and its contribution to the spectra was the highest for bran. The spectra of corn and white spelt flours could also be simulated by two signals, however, with higher g factors, $g = 2.0047$ and $g = 2.0052$ which indicated that signal with $g = 2.0041$ was characteristic of wheat bran structure. The spectra of white wheat and tapioca flours were similar and consisted of three signals (Fig. 6). Two of them (signal S-1 with $g = 2.0051$–2.0055, $A = 0.6$–0.8 mT and signal S-2 with $g = 2.0063$–2.0065, $A_1^H = 0.6 - 0.9$ mT, $A_2^H = 1.2$ mT) exhibited hyperfine splitting with parameters similar to those found for the respective heated starches (Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013), whereas the third signal, S-3, with the highest contribution to the spectrum (65–70%) was a single line at $g = 2.0048$. Signal S-1 was attributed to the radical localized at the C(1) carbon atom of the glucose unit in the starch matrix. This radical could be formed as a result of breaking of the α-1,4-glycosidic linkage caused by thermal treatment of starch. Signals of this kind were observed in the amylose and amylopectin fractions of corn, wheat and potato starch (Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013; Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Sepioło, 2013). Signal S-2 was found only in amylopectin and was ascribed to the radical created at the C(6) carbon atom (Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013; Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Sepioło, 2013). We postulated that such species were generated by thermal cleavage of α-1,6-glycosidic bonds. Initially, in both cases alkoxy radicals were created in addition to alkyl radicals, which subsequently abstracted hydrogen atoms from the neighbouring carbon atoms to produce carbon-centred radicals. The hyperfine splitting of signals S-1 and S-2 was caused by the interaction between magnetic moments of an unpaired electron and the nuclear spin of the β hydrogen atom in the S-1 centre and both α and β hydrogen atoms in the S-2 centre (Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013). Thermally treated potato flour gave a spectrum with also three signals: S-1 with $g = 2.0055$, $A = 0.5$ mT, S-2 with $g = 2.0066$, $A_1^H = 0.6$ mT, $A_2^H = 1.2$ mT and single line S-3 at $g = 2.0050$ with, however, significantly lower contribution than that found in the spectra of white wheat and tapioca flours (10%) (Fig. 6), The single line, analogical to S-3 but with low contribution was found in the spectra of various starches (potato, corn and wheat) and was attributed to the electron delocalized on conjugated double bonds in the

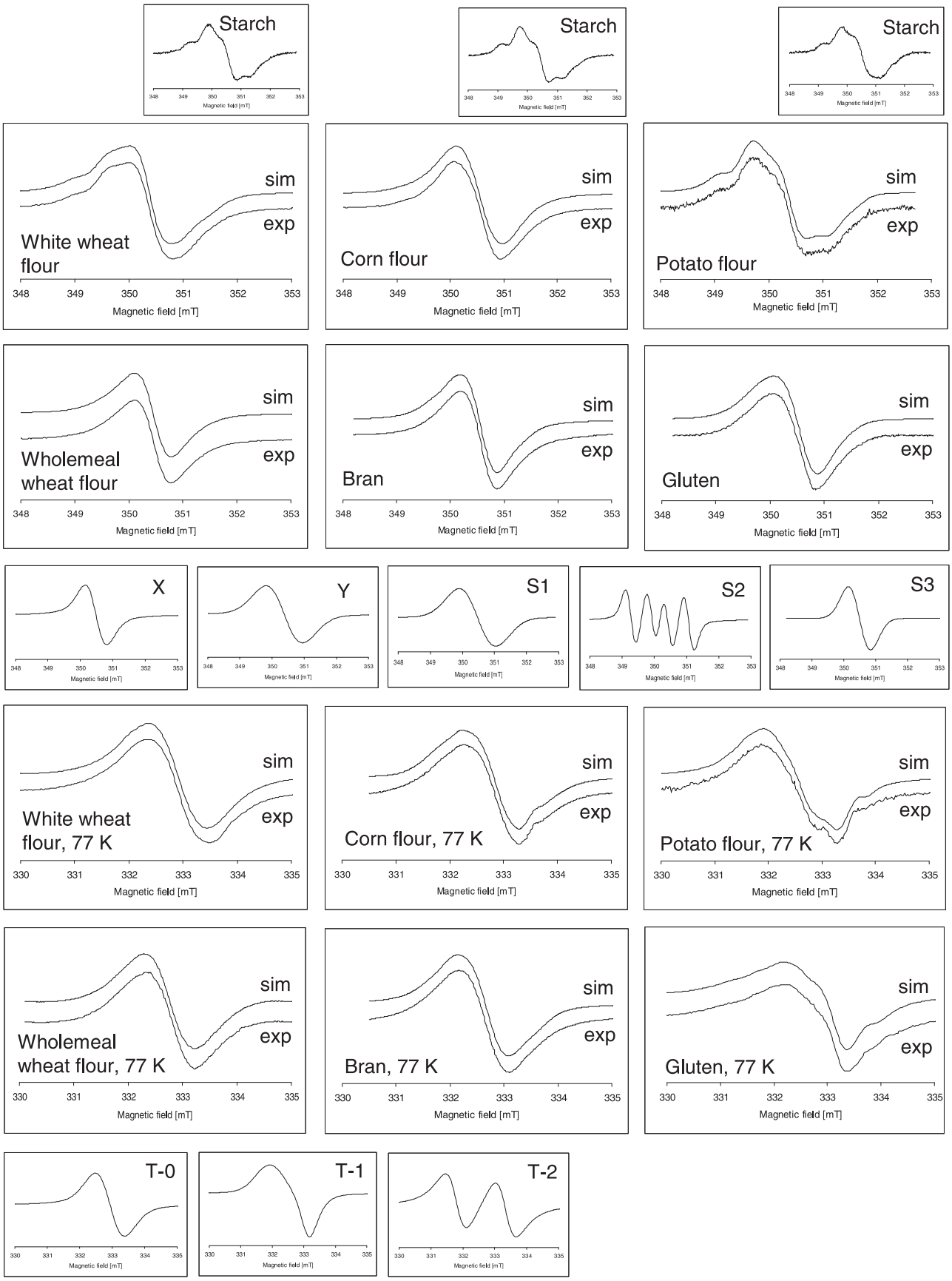

Fig. 6. The experimental and simulated spectra of thermally treated flours, gluten and bran recorded at room temperature and at 77 K in the range of 5 mT. Inserts: spectra of thermally treated respective starches, recorded at room temperature in the range of 5 mT. X, Y, S1, S2, S3 – particular signals used to simulate EPR spectra recorded at room temperature, T-0, T-1, T-2 – particular signals used to simulate EPR spectra recorded at 77 K.

structure of glucose ring reorganized upon heating (Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013; Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Sepioło, 2013). The significantly higher contribution of signal S-3 to spectra of flours (except potato flour) was correlated with the high total intensity of these spectra in comparison with those of starch. This phenomenon could result from the formation of similar radical species in the protein matrix present in the flour material and/or from the activation of the radical generation in the carbohydrate fraction of flours, occurring under the influence of transition metal ions (Fe, Cu) existing in flours. In our previous studies it was found that the intensity of radical signals strongly increased upon the introduction of transition metal ions (Cu, Fe) into the starch matrix. Moreover, the process was accompanied by the reduction of these metal ions (Łabanowska et al., 2008, 2011; Łabanowska, Kurdziel, et al., 2013). It was in line with studies of Lomnicki, Truong, Vejerano, and Dellinger (2008) who found that as a result of interaction of organic compounds containing substituted aromatic ring with Cu(II) ions, the reduction of metal species occurred with a simultaneous formation of stable organic radicals. Taking into account that the amounts of iron ions in white wheat and especially in tapioca flours were about four to nine times higher than those in potato flour, the latter mechanism should be considered. The activation of the formation of radicals could occur via the formation of transient reactive oxygen species able to remove hydrogen bonded to a carbon atom (Łabanowska et al., 2008, 2011; Łabanowska, Wesełucha-Birczyńska, Kurdziel, & Puch, 2013). Such active oxygen species R—C—O$^{\bullet}$ could be created as a result of breaking metal–oxygen bonds in metal–starch complexes which was accompanied by the reduction of metal ions. The second reason for the strong increase of the amount of radicals, especially in cereal flours, upon thermal treatment could be the result of the decomposition of proteins. Therefore gluten, the protein component of wheat flours was studied. In the spectrum of thermally treated gluten recorded at room temperature, two lines with $g = 2.0050$ and $g = 2.0040$ were found in the spectrum by simulation procedure. The stability of these signals and a lack of HFS originating from interaction with the nuclear spin of nitrogen suggested the presence of carbon-centred radicals. The g values of both signals in gluten were similar to those found for signals in flours. Signal S-3 in white wheat flour and signals with $g = 2.0052$ in both, corn and white spelt flours, could be counterparts of the signal at $g = 2.0050$, whereas signal X at $g = 2.0041$, present in the spectra of wholemeal wheat and spelt flours as well as bran could correspond to the signal at $g = 2.0040$. Hence, it could be suggested that some radical species in flours formed upon thermal treatment were situated in the protein matrix. Additional confirmation of this hypothesis was the low intensity of the spectrum of potato flour poor in proteins.

The process of radical formation in protein structures might be influenced by iron ions, present in plant tissues in both, organic and inorganic forms. Ferritin is the main storage centre of iron(III), where it occurs in oxide-hydroxides phase or is located in the protein shell in which iron atoms are connected by an oxygen bridge. These bridges could be broken upon thermal treatment with the formation of reduced Fe(II) ions and radical species of the FeO$^{\bullet}$ type, which could interact with protein residues leading to the formation of stable organic radicals of protein character, for example of the tyrosyl radical. This radical could also be formed by the transfer of an electron from tyrosine ligand to non coupled Fe(III) centre.

Similarly, as before thermal treatment, the number of radicals calculated from the integral intensity of spectra recorded at 77 K was two to four times higher than that found from the spectra recorded at room temperature. Simulation procedure revealed that spectra of tuber and corn flours consisted of signals with the EPR parameters characteristic of tyrosine radical and could be simulated by two signals: T-1, with parameters $g_1 = 2.0084$, $g_2 = 2.0043$, $g_3 = 2.0012$, $g_{av} = 2.0046$ and T-2 with $g = 2.0046$, $A^{H\beta} = 1.7$ mT. The anisotropic lines of signal T-1 and HFS lines of T-2 in the spectrum of corn flour were 2–3 times broader than those before heating and simultaneously HFS, resulting from electron coupling with ring protons, disappeared, which was caused by an increase of dipole-dipole interactions between radical species whose content was higher than before heating (Fig. 2). In the spectrum of tuber flours signals T-1 and T-2 manifested themselves only after heating. The parameters of signals in the spectrum of heated gluten were slightly different: $g_1 = 2.0097$, $g_2 = 2.0049$, $g_3 = 2.0025$ ($g_{av} = 2.0057$) for the first signal and $g_{av} = 2.0057$, $A_2^{H\beta} = 2.2$ mT for the second signal. The simulation of the spectra of bran, wheat and spelt flours (white and wholemeal) showed that the main signal of the spectrum was the isotropic line T-0 with $g = 2.0045$, similar to g_{av} of the tyrosyl signal and the same as that found for signal Y recorded at room temperature. The contributions of the anisotropic line of tyrosyl radical T-1 with $g_1 = 2.0076$, $g_2 = 2.0047$, $g_3 = 2.0010$, $g_{av} = 2.0045$ and the doublet T-2 with $g = 2.0044$, $A^{H\beta} = 1.5$ mT were about 20–30% and 4–5%, respectively. Such changes in EPR parameters of the signals could be correlated with the strong increase of the amount of radicals in these flours and bran and were caused by a strong coupling between radicals leading to a decrease of anisotropic and hyperfine interactions and the appearance of isotropic line. The formation of the tyrosyl radical upon thermal treatment could occur in two ways: by the transfer of an electron from the tyrosine molecule to a transition metal ion – Fe(III) or Cu(II), or by the abstraction of a hydrogen atom from the hydroxyl group of tyrosine by reactive oxygen species formed with the participation of transition metal ions. In the former process tyrosyl cationic radicals were created, whereas the latter provided neutral tyrosyl radicals. In both processes the formation of radical species was related to reduction of Fe(III) and/or Cu(II). In our considerations we excluded the change of Mn(II) to Mn(I) as a rather unlikely process and oxidation to Mn(III), which is EPR silent, was in our opinion a reason for the reduction of the Mn(II) signal intensity upon thermal treatment.

5. Conclusions

Analysis of the spectra of flours revealed that radicals existed not only in thermally treated materials, but also in native samples of flours, especially in cereal wholemeal flours. The main source of radicals in untreated material was bran, present in these flours. Radicals were mostly of phenoxyl and semiquinone origin; however, the small amounts of carbon-centred radical species should be also taken into account. The phenoxyl radical was situated at the tyrosine moiety, whereas semiquinone radicals were formed only in bran upon oxidation of phenolic compounds during drying of grains. The increase of the amount of radicals upon thermal treatment correlated with the strong decrease of the intensity of Fe(III) signals in all flours and bran, suggested an interdependence of both processes. Additionally, it was noticed that significantly higher amounts of radicals were generated in materials rich in these metal ions. Hence, we postulate that formation of radicals in flours is influenced by changes in the redox status of the transition metal ions – protein/carbohydrate molecules systems. These changes can occur by the electron transfer from tyrosine to these ions and by the formation of transient reactive oxygen species with the participation of transition metal ions. Such oxygen species can detach hydrogen atoms from carbon atoms of carbohydrates and/or proteins and from hydroxyl group of tyrosine moiety. As a result, stable carbon-centred radical species and tyrosyl radicals are formed. It seems that the presence of Fe(III) ions significantly facilitates generation of radicals regardless of the type of radicals and the matrix of their stabilization. In potato flour, poor in transition metal ions but containing high amount of starch, the number of thermally created radicals is lower and radicals are formed mostly in starch

structures, suggesting the operating mechanism is that assumed for native starch.

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