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ORIGINAL PAPER

Antioxidant activity of cookies and its relationship with heat-processing contaminants: a risk/benefit approach

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Abstract Formation of both health promoting and potential harmful substances (acrylamide and hydroxymethylfurfural) has been associated to the extent of the Maillard reaction. The effects of recipe compositions in terms of leavening agent (ammonium and sodium bicarbonates) and sugars (sucrose and glucose), and baking conditions (temperature and time) on the antioxidant activity (AOA) in cookies were studied. The cookies were baked at different temperatures (180–220 °C) for different times (10–25 min). AOA was measured by the ferric reducing power (FRAP), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2,-diphenyl-1-picrylhydrazyl (DPPH) coloured radicals, and oxygen radical absorbance capacity (ORAC-fluorescein) in automated plate-reader assays. Net AOA varied regarding the assay applied, whereas higher AOA was always obtained for the ABTS assay and lower for DPPH assay, and ranging from 2 to 200 μmol Trolox/g sample. At higher temperature and baking times, higher AOA in cookies regardless of the formulation was recorded. Glucose enhances formation of compounds with higher AOA compounds as compared with sucrose recipes. Ammonium bicarbonate clearly promotes the formation of AOA for sucrose recipes but this effect is not observed in glucose recipes and varied with the AOA procedure applied. A risk/benefit index, based on the concomitant formation of neo-formed contaminants and substances with AOA (potentially health-promoting substances) is presented, and its application for recipe comparison is discussed. Risk/benefit index rapidly increased with increased temperature and time of baking.

Keywords Maillard reaction · Acrylamide · Hydroxymethylfurfural · Antioxidant activity · Cookies · Baking · Risk/benefit index

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Introduction

Maillard reaction products (MRP) are associated with some positive biological actions, such as antioxidant activity (AOA) [1], chemopreventive activity [2], anticarcinogenic [3], antimutagenic [4], antitumoral properties [5], antihypertensive activity [6], and prebiotic [7], among others. As reviewed by Henle [8], specific MRP in foods may even act as chemoprotective agents, like melanoidins [9, 10], pronyllysine [11], and *N*-methylpridinium, a low-molecular constituent formed during roasting of coffee, which increased total antioxidant capacity in the plasma of rats fed with coffee melanoidins [12].

But simultaneously MRP are known to induce negative consequences; among them are antinutritional properties (impairment of amino acids and protein crosslinking), loss of soluble vitamins and availability of minerals, apart from some mutagenic and carcinogenic actions have also been associated to MRP [2, 13]. Acrylamide (ACR) has been classified as a possible carcinogenic to humans [14]. Although the toxicological relevance of hydroxymethylfurfural (HMF) is not clear as in vitro studies on genotoxicity and mutagenicity have given controversial results [15, 16]; its presence is not recommended in foods and it is under evaluation. HMF and acrylamide are naturally formed during baking of cookies.

The depletion of the natural occurring antioxidants in processed foods could be balanced by the formation of novel compounds with antioxidant activity [17]. Then, by optimizing the industrial processing conditions, it could be possible to keep the overall antioxidant activity by enhancing the formation of certain MRP with antioxidant properties. MRP influence the oxidative stability and shelf-life of several foods [18]. The AOA of MRP is influenced by several factors: ratio and type of amino compounds and sugars, temperature, pH, and water activity (i.e. 5, 19, 20, 21). It has been hypothesised that the main mechanism of action is the ability of trapping positively charged electrophilic metabolites; scavenge oxygen radical, metal chelation or synergism (i.e. 22, 23). However, the compounds accounting for this effect have not been clearly identified and the mechanism of antioxidant activity is still under study.

Effect of natural antioxidants on the formation of acrylamide is controversial. Rydberg et al. [24] used ascorbyl palmitate and sodium ascorbate in a potato model for baking, and suggested that the involvement of radicals or peroxidation in the formation of acrylamide is minor. Furthermore, Tareke et al. [25] described that the addition of antioxidants to meet before heating induced the formation of acrylamide via protection of its degradation from radical initiated reactions. However, Zhang et al. [26] demonstrated that acrylamide was significantly reduced (up to 76%) in fried potatoes, fried chicken and fried bread sticks when dipped into different solutions of bamboo leaves antioxidants. At the same line, acrylamide is reduced in fried potato when adding rosemary herb to the frying oil [27] or with addition of spice extracts [28]. Recently, Napolitano et al. [29] have clearly shown the influence of the ortho-diphenolic composition of the frying oil on the formation of acrylamide.

In previous studies, we have shown the formation of HMF and ACR during baking of cookies [30, 31] but relationship with health-promoting factors, such as AOA, were not considered. Recently, Summa et al. [32] discussed the effect of the mitigation strategies adopted to minimise acrylamide formation during baking on the antioxidant properties of cookies. Authors stated that any mitigation effort must also take into account the loss of potentially beneficial constituents to health and consequently changes in consumer perception. In fact risk/benefit evaluation of thermally processed foods is an important issue that it should be clarified in future investigations dealing with new formed contaminants.

The aim of the present paper is to investigate the effects of recipe compositions in terms of leavening agent and sugars, and baking conditions on the AOA in cookies. AOA activity is measured by different approaches to give a broader understanding on the activity of water soluble substances formed during baking of cookies. Risk aspects, formation of potentially harmful compounds and benefit aspects, formation of processing antioxidants are discussed.

Material and methods

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein disodium, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and potassium persulphate, sucrose, glucose, ammonium bicarbonate were purchased from Sigma (St. Louis, MO, USA). 2,2′-azobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) were from Fluka Chemicals (Madrid, Spain). Hydroxymethylfurfural and acrylamide were purchased from Sigma (Diesenhofen, Germany). Iron (III) chloride, sodium phosphate monobasic, sodium bicarbonate, hydrogen peroxide, sodium chloride, chloroform, and hydrochloric acid were purchased from Panreac (Madrid, Spain). Methanol, acetonitrile, glacial acetic acid, potassium hexacyanoferrate and zinc sulphate were purchased from Merck (Darmstadt, Germany). [$^{13}C_3$]-acrylamide (isotopic purity 99%) was from Cambridge Isotope Labs (Andover, MA, USA). Atlantis dC18 (300 × 4.6 mm, 5 μm) column and HLB-cartridges were supplied by Waters (Milford, MA, USA). Micro-spin PVDF centrifuge filters (0.45 μm) were purchased from Alltech (Deerfield, IL, USA). Inertsil ODS-3 column (250 × 4.6 mm, 5 μm) was supplied by GL-Sciences INC (Tokyo, Japan) and ZORBAX Bonus-RP, Narrow Bore RR 2.1 × 100 mm 3.5 μm) was supplied by Agilent Technologies (Wilmington, DE). 96-well microplates were from Biogen Científica (Madrid, Spain.

Preparation of cookies

Flour and shortening were supplied by local producers, and other ingredients were purchased from local supermarkets. Reference cookies were prepared according to five recipes as given in Table 1. Control dough was prepared according to a recipe described in AACC (American Association of Cereal Chemists) methods 10–54 [33]. The amount of wheat flour, shortening, salt and water were fixed, while the types of leavening agents (ammonium bicarbonate and sodium bicarbonate) and sugars (sucrose and glucose) were selected as the variables in the recipes which would have certain effects on the AOA of cookies. The dough was rolled out to discs with the diameter of 5.5 cm and the height of 2 mm, and baked at different temperatures (180–220 °C) for different times (10–25 min). A natural

Table 1 The composition of recipes used to prepare cookies

Ingredient	Amount in the dough (g)				
	Reference	SNH4	SNA	GNH4	GNA
Wheat flour	80.0	80.0	80.0	80.0	80.0
Shortening	32.0	32.0	32.0	32.0	32.0
Salt	1.0	1.0	1.0	1.0	1.0
Deionised water	17.6	17.6	17.6	17.6	17.6
Leavening agents					
Sodium bicarbonate	0.8	0.0	1.2	0.0	1.2
Ammonium bicarbonate	0.4	1.2	0.0	1.2	0.0
Sugars					
Sucrose	35.0	35.0	35.0	0.0	0.0
Glucose	0.0	0.0	0.0	35.0	35.0
Dough pH value[a]	8.28	8.14	7.83	8.41	8.42
pH at 220 °C for 25 min[a]	7.45	5.10	7.74	5.56	5.36

[a] Expressed in pH value

convection oven (Labor-Simsek, Turkey) was used for baking trials.

Analysis of hydroxymethylfurfural

The analysis of HMF was performed as described by Gökmen and Şenyuva [34]. Finely ground sample (1 g) was mixed with 100 µL of Carrez 1 and 100 µL of Carrez 2 solutions was added, and the volume was completed to 10 mL with 0.2 mM acetic acid. After double extraction and centrifugation (5,000 rpm for 10 min at 0 °C), supernatant was cleaned up further by using an Oasis HLB SPE cartridge and eluted with diethyl ether. The eluate was collected in a conical-bottom glass test tube placed in a water bath at 40 °C (Zymark Turbo Vap LV evaporator) and evaporated to dryness under nitrogen at 3 psig. The remaining residue was immediately redissolved in 1 mL of water by mixing in a vortex mixer for 1 min. The chromatographic separations were performed on an Zorbax Bonus-RP Narrow Bore column (2.1 × 100 mm, 3.5 µm) using the isocratic mixture of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid at a flow rate of 0.2 mL/min at 40 °C. Data acquisition was performed in selected ion monitoring (SIM) mode and with an atmospheric pressure chemical ionisation (APCI) interface. Ions monitored for HMF were *m*/*z* 109 and 127. The quantification was performed on the basis of the signal response of the ion having *m*/*z* of 109. Chromatograms were also monitored at a detection wavelength of 285 nm.

LC–ESI-MS determination of acrylamide

A sample preparation as described by Şenyuva and Gökmen [35] was used, and analysis was as described by Morales et al. [36] using an Agilent 1100 HPLC system (Waldbronn, Germany) coupled to an Agilent 1100 MS detector equipped with an electrospray ionisation interface. The analytical separation was performed on an Inertsil ODS-3 column (250 × 4.6 mm, 5 µm) using an isocratic mixture of 0.2% aqueous solution of formic acid at a flow rate of 0.6 mL/min at 25 °C. Data acquisition was performed, with a delay time of 8 min, in a selected ion monitoring (SIM) mode using the following interface parameters: a drying gas (N_2, 100 psig) flow of 12 L/min, nebulizer pressure of 45 psig, drying gas temperatures 350 °C, a capillary voltage of 3 kV and a fragmentator voltage of 70 eV. Monitored ions were 72.1 *m*/*z* for acrylamide and 75.1 *m*/*z* for $^{13}C_3$-labeled acrylamide. An acrylamide calibration curve was built in the range of 2–100 µg/L and a limit of quantitation was determined at 25 µg/kg for potato crisp. The method was in-house validated for linearity, precision and recovery. Precision (reproducibility) was lower than 12% and recovery between 84–109%. Furthermore, the accuracy was recently demonstrated for potato crisps in an interlaboratory comparison study (IRMM 2008) [37], yielding a *z*-score of −0.5. The analyses are integrated within the scope of a certified laboratory controlled by AENOR (Spanish Association for Standardisation and Certification).

Measurement of pH

Ground sample (0.4 g) was mixed with 20 ml of water and vortexed for 3 min. The mixture was held at ambient temperature for 1 h to separate solid and liquid phases. pH was measured after appropriately removing the supernatant layer by using a pH meter (CG-837 pH meter, Schott, Mainz, Germany).

Sample extraction for antioxidant assay

Sample (1 g) was extracted in 5 mL of deionised water (at 40–50 °C) in a 10-mL centrifuge tube. The tube was shaken vigorously for 5 min and stand for 30 min at 4 °C. The mixture was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was collected in a flask and two additional extractions were performed using 2 mL of prewarmed deionised water. The supernatants were pooled, filtered (0.45 µm) and stored at −80 °C until analysis.

Antioxidant assays

A Synergy™ HT-multimode microplate reader with automatic reagent dispense, and temperature control from Biotek Instruments (VT, USA) were used. Biotek Gen5™ data analysis software was used. Each 96-wells plate was designed to contain four repetitions per sample and

standard, four levels of standards for calibration, and eight repetitions per blank or control. After that, reaction was started by automatic addition of 60 μL of FRAP solution, ABTS radical cation solution, DPPH radical or AAPH, for FRAP, ABTS, DPPH and ORAC assays, respectively.

DPPH assay

Procedure was based on that reported by Delgado-Andrade et al. [38] for MRPs and adapted to a plate reader. 40-μL aliquot of test (methanol, Trolox, or sample) and 200-μL methanol were placed in a well of a 96-well microplate. Sample was previously diluted in water (1/2, 1/10, 1/20, and 1/40) accordingly. Plate reader automatically dispensed 60 μL of DPPH solution. Methanolic solution of DPPH (690 mg/L) was daily-prepared and absorption at 520 nm was checked to be 1.8 ± 0.02 AU for a tenfold diluted solution in a cuvette spectrophotometer (Shimadzu UV–visible 1601 spectrophotometer, Duisburg, Germany). Mixture was shaken before readings. DPPH absorption decay was recorded every minute at 520 nm for 60 min until a plateau was recorded. Temperature in the measurement chamber was set at 30 °C. The antiradical activity of sample was expressed disappearance of the initial purple colour; then the higher the disappearance, the greater the antiradical activity. Trolox solutions (50, 100, 200, and 300 μM) were used for calibration in every experiment/plate. Sample absorbance, after blank subtraction, at 60 min of reaction was substituted in the Trolox calibration curve. All the reaction mixtures were prepared in duplicate, and four independent assays were performed for each sample. Limit of quantitation was 10 μM. The results were expressed as micromolar equivalents of Trolox/g of sample.

$ABTS^{+\cdot}$ assay

The antioxidant capacity was estimated in terms of radical-scavenging activity, following the procedure described by Delgado-Andrade et al. [38] for MRP and adapted to a plate reader. Briefly, the blue-green $ABTS^{+\cdot}$ was produced by reacting 7-mM ABTS stock solution with 2.45-mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+\cdot}$ solution (stable for 2 days) was diluted with 5-mM phosphate buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. 40-μL aliquot of test (buffer, Trolox, or sample) and 200-μL phosphate buffer were placed per well in a 96-well microplate. Plate reader automatically dispensed 60 μL of $ABTS^{+\cdot}$ solution. Readings at the absorption maximum (730 nm) were taken every minute using a microplate reader thermostated at 37 °C and the reaction was monitored up to 30 min. Net sample absorbance at 6 min was substituted in the calibration curve. Calibration was performed, as described previously, with Trolox standard solutions (50, 100, 200, and 300 μM). Sample was previously diluted (1/2, 1/10, 1/20, and 1/40) in buffer accordingly. All the reaction mixtures were prepared in duplicate, and four independent assays were performed for each sample. Limit of quantitation was set at 40 μM. Results were expressed as micromolar equivalents of Trolox/g of the sample.

FRAP assay

The ferric reducing ability was estimated according to the procedure applied by Delgado-Andrade et al. [38] for MRPs and adapted to a plate reader. 40-μL aliquot of test (buffer, ferric chloride, or sample) and 200-μL acetate buffer (0.3 M, pH 3.6) were placed per well in a 96-well microplate. Plate reader automatically dispensed 60 μL of a prewarmed FRAP solution at 37 °C. Calibration was performed with ferric chloride stock solutions (25, 100, 250, and 500 μM). Sample was previously diluted (1/2, 1/10, 1/20, and 1/40) accordingly. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40-mM HCl plus 2.5 mL of 20-mM $FeCl_3 \cdot H_20$ and 7.5 mL of 0.3 M acetate buffer, pH 3.6. Readings at the absorption maximum (595 nm) were taken every 15 s using a microplate reader thermostated at 37 °C and the reaction was monitored up to 30 min. Sample was previously diluted (1/2, 1/10, 1/20, and 1/40) in buffer accordingly. All the reaction mixtures were prepared in duplicate, and four independent assays were performed for each sample. Limit of quantitation was set at 10 μM. Results are expressed as micromolar equivalents of ferric chloride/g of sample.

ORAC assay

$ROO^{\cdot}$ scavenging activity was measured by monitoring the fluorescence decay as result of $ROO^{\cdot}$-induced oxidation of fluorescein, known as the oxygen radical absorbance capacity (ORAC) assay. The water soluble azo initiator AAPH was applied as a clean and controllable source of thermally-produced alkylperoxyl free radicals ($ROO^{\cdot}$) in aqueous media. $ROO^{\cdot}$ are generated by AAPH in a microplate reader at 37 °C. The antiradical activity against AAPH was estimated according to the procedure reported by Huang et al. [39] slightly modified as Dávalos et al. [40]. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Samples (previously diluted at 1/2, 1/5, 1/10, 1/20 and 1/40) or Trolox (20 μL) and fluorescein (120 μL; 70 nM, final concentration) solutions were placed in each well of a black 96-well polystyrene microplate. The mixture was pre-incubated for 15 min at 37 °C on the microplate

reader. AAPH solution (60 μL; 18 mM, final concentration) was added rapidly using the plate reader dispenser, the microplate was shaken for 15 s and the fluorescence was recorded every minute for 90 min at 485 and 528 nm excitation and emission wavelengths, respectively. A blank (fluorescein + AAPH) using phosphate buffer instead of the sample solution and eight calibration solutions using Trolox (10, 20, 30, and 40 μM, final concentration) as antioxidant were also carried out in each assay. Limit of quantitation was set at 5 μM. All the reaction mixtures were prepared in duplicate, and four independent assays were performed for each sample.

Raw data were processed by the microplate reader and the area under the curve (AUC) was calculated according to the equation

$$\mathrm{AUC} = F_0/F_0 + F_0/F_1 + F_0/F_2 + \cdots + F_0/F_n$$

with F_0 = fluorescence intensity at time 0, F_1 = fluorescence intensity at time 1 min, and so forth. Finally, the net AUC was calculated by subtracting the AUC of the blank sample to the AUC of each sample. The effects are expressed as the relative Trolox equivalent ORAC value, which is calculated by the equation: relative ORAC Value = $[(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}})/(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}_{\mathrm{blank}})] \times$ (molarity of Trolox/g sample), where AUC is the area under curve. ORAC values were expressed as Trolox equivalents by using the standard curve calculated for each assay and final results were expressed in micromolar equivalents of Trolox/g of sample.

Statistical analysis

The statistical analysis was performed by ANOVA following a simple, balanced one-way model. The Duncan *t* test was used to compare the means and the levels of significance was set at 95%. Statgraphics v.2.0 software (Statistical Graphics Corp, Rockville, MD) was used.

Results and discussion

A large number of methods have been described in literature to measure the efficiency of antioxidants in foods extracts as an index of the antioxidant status in vivo. These methods are based on different mechanisms of the antioxidant defence system, i.e. scavenging of oxygen and hydroxyl radicals, reduction of lipid peroxyl radicals, inhibition of lipid peroxidation, or chelation of metal ions. In the present investigation, different approaches for in vitro estimation of the AOA (ferric reduction and antiradical activity) were applied to obtain a broader view of the changes in the AOA during baking of cookies. The study is focused on the formation of water soluble heat-induced substances which are representative of the extent of the baking process and, subsequently, markers of the different reactions taking place.

Method performance

In a first step, in vitro AOA assays were previously adjusted to an automatised plate-reader approach. Repeatability (coefficient of variation, $n = 8$) was 1.1, 3.3, 2.2, and 1.5% for ABTS, DPPH, ORAC, and FRAP assays, respectively. Reproducibility (day-to-day) was lower than 12.2% for all AOA assays giving a good precision. A Trolox standard solution of 100 μM was used for ABTS, and DPPH, 20 μM for ORAC assay, a 250 μM of $FeSO_4$ for FRAP assay, and a cookie sample was selected as reference and was used to evaluate precision.

Effect of baking conditions on AOA

The effect of baking temperature and time on the AOA of cookies was studied. Recipe designed as reference was selected to illustrate the results, and rest of the recipes had a similar trend. Figure 1 depicted the results obtained for different antioxidant assays; FRAP (a), ABTS (b), DPPH (c), and ORAC (d). Results are expressed as mole of the reference substance used for calibration (Trolox or $FeSO_4$)/g of sample. As expected, AOA of cookies increase as the baking temperature and time increased regardless of the method applied for AOA measurement. However, AOA for each assay was different being always higher for the ABTS method. These differences are expected by the different chemistry that lies behind each AOA assay.

In the FRAP method, the yellow Fe^{3+}-TPTZ complex is reduced to the blue Fe^{2+}-TPTZ complex by electron-donating substances under acidic conditions. Any electron-donating substance with a lower redox potential than Fe^{3+}/Fe^{2+}-TPTZ will drive the reaction and the formation of the blue complex forward. Formation of ferric reducing substances becomes significant at extended baking times (25 min) at all temperatures, but AOA due to ferric reducing capacity were not significant at baking time lower than 20 min for 180–210 °C (Fig. 1a). Similar results were obtained for the ABTS assay, which the decolourisation of the ABTS radical cation is also based on electron transfer capacity (Fig. 1b). The redox potential of Fe(III) salt (aprox. 0.70 V) is comparable to that of ABTS radical (0.68 V).

Estimation of the AOA by scavenging of stable radicals such as the chromogen radical DPPH in inorganic media has been extensively used for comparison of homogeneous series of antioxidants. This procedure measures the hydrogen donating capacity of the target substances in a methanolic media. The colour changed from purple to

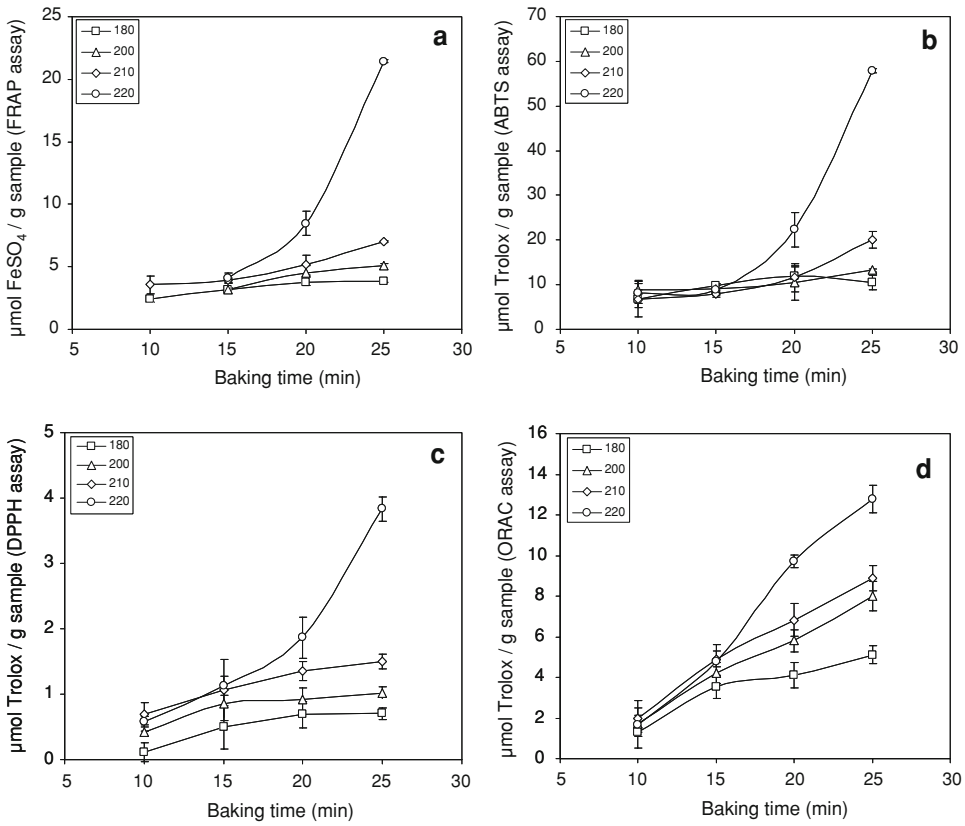

Fig. 1 Effect of temperature and time of baking in the reference recipe on the AOA as measured by FRAP (**a**), ABTS (**b**), DPPH (**c**) and ORAC (**d**) assays

yellow by acceptance of a hydrogen radical from MRP and it became a stable diamagnetic molecule. Then, the decrease in DPPH radical indicated the radical-scavenging activity of the MRP formed during baking. Figure 1c depicted results of control cookie for AOA measured by the DPPH assay. Significant differences among recipes were obtained for 25 min of baking time. It was not observed a lag phase and presence of water soluble substances with AOA was already detected at 10 min of baking.

Finally, ORAC is based on quenching of fluorescence from fluorescein by short-lived radicals. Peroxyl radicals, which are intermediates in lipid oxidation, are generated by azo compounds such as AAPH. This procedure is considered to be more realistic to the antioxidant mechanism occurring in living bodies. The reactivity of sugars in the dough depends on their physical state and water content during baking. At the beginning they are dissolved and further they crystallise to form an amorphous glass state (Fig. 1d). This finding is relevant since those water soluble compounds in cookies accounting for AOA could act in the gastrointestinal tract or could be physiologically absorbed and exert their activity in vivo. Studies have revealed that the MR was closely related to several degenerative diseases such as diabetes and aging [41]. For example, enzyme forms of protein accompanied by glucose trigger the MR, and MRP are known to produce active oxygen species and to possess scavenging activity on them [42].

In light of these results, it cannot be concluded if increase of AOA of water soluble fraction of cookies is due to a specific group of substances chemically similar or to the formation new substances with more powerful AOA at the advance stage of the MR. AOA measured by DPPH assay was lower whether compared with FRAP, ABTS, and ORAC assays. The difference is due to the fact that some components, making an important contribution to the AOA of the aqueous dilutions, were not soluble in methanol. Probably the fact that DPPH method was developed in methanolic media was responsible of the low response found.

Effect of formulation on the AOA

Four different recipes (SNH4, SNA, GNH4, GNA) were formulated varying the type of sugar (glucose or sucrose) and leavening agent (sodium bicarbonate or ammonium bicarbonate) but keeping constant their relative amount in the formula. According to the former AOA results on the cookie recipe used as reference, 25 min of baking time was selected as reference for 180, 200, 210, and 220 °C to illustrate the changes. Figure 2 depicted the results obtained for FRAP (a), ABTS, (b), DPPH (c), and ORAC

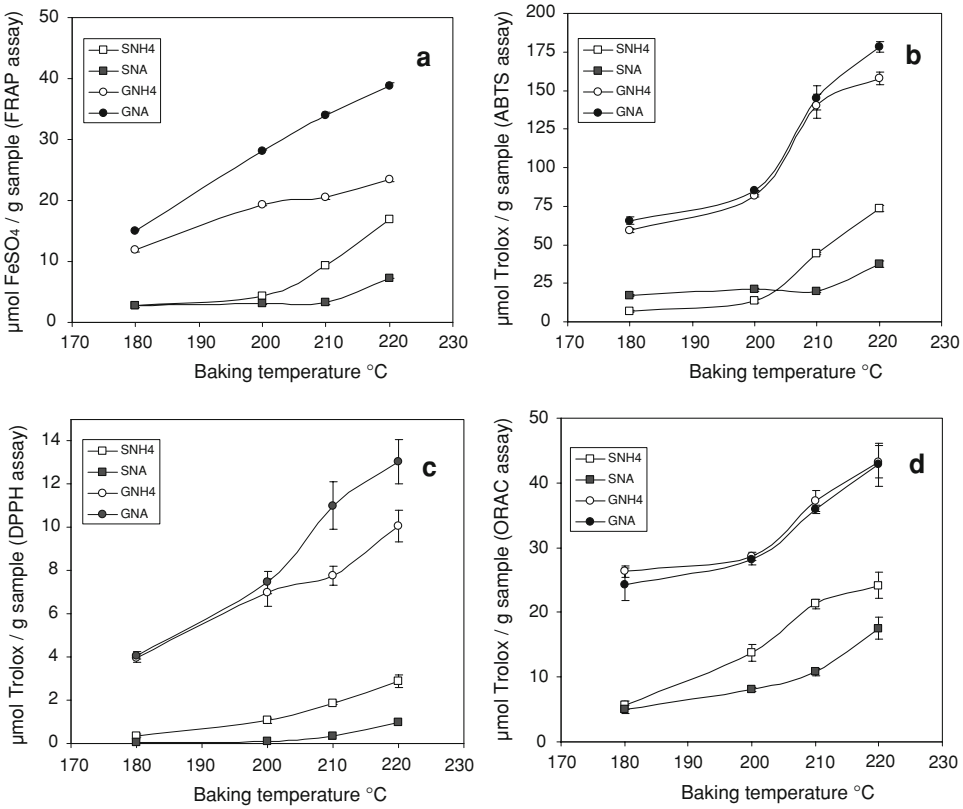

Fig. 2 Effect of formulation of cookies baked at 180, 200, 210, and 220 °C for 25 min on the AOA as measured by FRAP (**a**), ABTS (**b**), DPPH (**c**) and ORAC (**d**) assays

(d) in cookies baked at 180, 200, 210, and 220 °C for 25 min.

AOA of cookies formulated with glucose (GNH4 and GNA) were significantly higher than sucrose (SNH4 and SNA) formulated recipes for all the antioxidant assays tested. This effect can be first explained by the higher reactivity of glucose in both MR and sugar caramelisation reactions. Formation of water soluble substances with AOA is increasing linearly with the time of baking without showing a lag phase. During heating, sucrose and starch may hydrolyse into glucose and fructose, respectively or a mixture of maltodextrin, maltose and glucose. The newly formed maltose and monosaccharides are reducing sugars which can further participate in both the MR and in the caramelisation. When sucrose was replaced with glucose in the dough, AOA of cookies increased rapidly upon onset of baking.

In addition, the effect of two leavening agents (ammonium bicarbonate and sodium bicarbonate) was studied. Organic acids, such as citric acid or tartaric acid, are often added to baking agents containing $NaHCO_3$ to enhance leavening [44]. There are some differences in the effect of leavening agent on AOA when glucose and sucrose recipes are compared. Presence of ammonium bicarbonate enhances formation of water soluble substances with higher AOA in sucrose recipes, but no differences (for ABTS and ORAC) or even an opposite (for FRAP and DPPH) behaviour was observed in glucose recipes as compared with sucrose ones. There is a significant difference between the pH values of cookies made of different recipes as influenced by the type of leavening agent. In cookies formulated with sucrose and sodium bicarbonate (SNH4) and baked at 220 °C for 25 min, the pH value was 5.1 (Table 1). Use of ammonium bicarbonate was characterised by an increase in the acidity of cookies (SNH4, GNH4) reaching pH levels about 5.0 at the end of baking. However, recipe GNA formulated with sodium bicarbonate and glucose showed a pH value close to 5.0, as well. Presence of glucose in the formulation will increase the rate of both sugar caramelisation and subsequent degradation to acids, mainly formic acid, but also Maillard reaction in enhanced where formation of acetic acids is the predominant [45].

Evaluation of the risk/benefit

Baking of cookies not only promote the formation of substances with antioxidant activity but also potentially harmful ones. HMF and acrylamide are considered neoformed heat-induced contaminants in thermally processed food. HMF and ACR were previously measured in the recipes. Negative aspects of baking (formation of potentially harmful compounds) were compared to positive aspects of baking (formation of substances with antioxidant

activity) with the aim to get a picture of the overall situation. Since four different assays for measuring AOA was applied, independent results in each baking combination were harmonised to the highest value obtained in every trial. For instance, 44.4 μmol Trolox equivalent/g sample was obtained for recipe SNH4 baked at 210 °C for 25 min as measured according to ABTS assay (AOA_{ABTS}). The highest value of AOA_{ABTS} assay was obtained in the recipe GNA baked at 220 °C for 25 min, 178.2 μmol Trolox equivalent/g sample. Then, the relative AOA_{ABTS} is 24.9%. Same harmonisation was also done for DPPH, FRAP, and ORAC assays. For each recipe and combination of temperature and time of baking, a relative AOA_{ABTS}, AOA_{DPPH}, AOA_{FRAP}, and AOA_{ORAC} was calculated and it represents the positive aspect of baking. Risk/benefit index was calculated by dividing the concentration of HMF (mg/kg sample) or ACR (μg/kg sample) by the average AOA obtained by different approaches. This index is useful just to compare changes in recipes and baking conditions but it is strongly limited to explain biological effects since we are not able to evaluate the impact of both parameters in vivo.

Risk/benefit index was applied to evaluate the effect of baking. Figure 3a represents the relative formation of HMF and antioxidants during baking in the reference recipe. Risk/benefit index dramatically increased at more severe baking conditions, showing a net increase of the risk with increased baking conditions. Overall AOA activity of cookies is enhanced with increasing temperature and time of baking but it is not balanced to the formation of HMF, being more than twofold the risk/benefit index obtained when baked at 210 °C. In other words, increase of AOA during baking did not compensate the strong formation of HMF at more severe baking conditions. However, risk/benefit index associated with acrylamide showed a different behaviour (Fig. 3b). Risk/benefit index increased with temperature and time but differences between baking temperatures are not significant between 20 and 25 min.

Effect of formulation on the risk/benefit index was also evaluated. Our previous investigation described that levels of acrylamide and HMF are closely related to the baking conditions and formulation [30, 31]. During baking, dough mainly undergoes changes in structure, taste, colour, and size, whereas MR and water evaporation are important. It has been reported that the addition of acids, by means of lowering pH, decreased the amount of acrylamide formed in foods during heating (i.e. 46). The protonation of the α-amino group of asparagine hinders the formation of the *N*-substituted glycosylamine, which may explain the reduced acrylamide content. In a similar way, Gökmen et al. [30] reported that presence of ammonium bicarbonate in recipes formulated with sucrose increase significantly the formation of HMF. According to Kroh [47], at neutral–basic pH, the MR

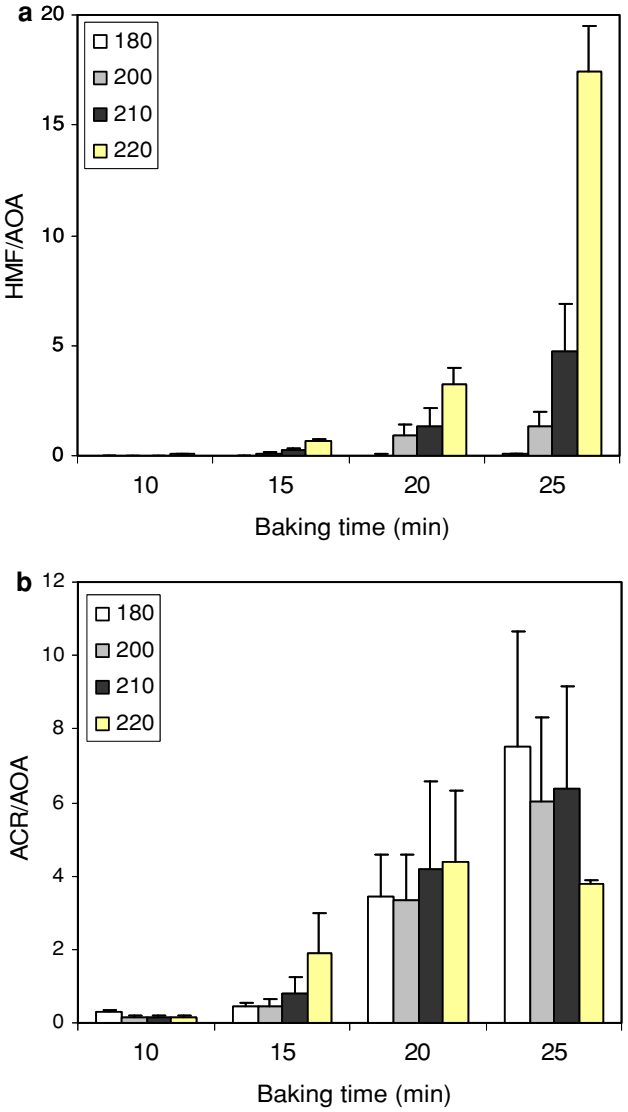

Fig. 3 Effect of the temperature and time of baking in the reference recipe on the risk/benefit index for hydroxymethylfurfural/AOA (**a**) and acrylamide/AOA (**b**)

reaction is more rapid than the caramelisation, which is favoured at acidic pH and higher temperatures.

Figure 4a depicted results of the risk/benefit index associated to HMF and obtained for recipes with different combination of sugars and leavening agent taking all data at once. Data were newly harmonised to the highest value obtained for the set of samples in order to be compared among them (temperature, time, and recipe). Recipe SNH4 showed the significantly highest risk/benefit index indicating that formation of substances with antioxidant activity did not compensate the formation of HMF as compared with other formulations. Recipe SNA showed a significantly higher risk/benefit index for acrylamide (Fig. 4b). These results are controversial since it is reported in the literature that acrylamide is enhanced in the presence

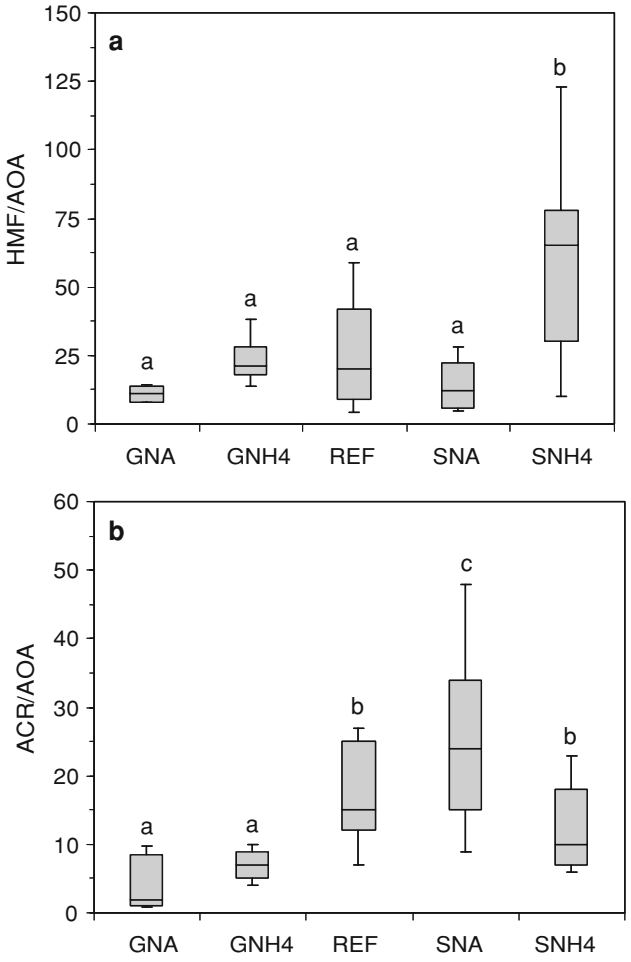

Fig. 4 Overall effect of formulation of five cookie recipes on the risk/benefit index for hydroxymethylfurfural/AOA (**a**) and acrylamide/AOA (**b**). Different letters shows statistically significant groups

of ammonium bicarbonate during baking. But, on the other hand, ammonium bicarbonate was not efficient to enhance the formation of substances with antioxidant activity.

As described by Summa et al. [32], strategies for the mitigation of new formed processing contaminants in foods, go directly to the mechanism of the MR by limiting its extent, and subsequently, the formation of both health-promoting and harmful compounds. MR is an extremely complex cascade of reactions where a myriad of compounds are formed with, sometimes, opposite functional properties. Nowadays, it is a challenge to drive the reaction to enhance formation of positive/healthy substances and consequently minimise the formation of negative/harmful ones. AOA is one of the functional properties of MRP. Therefore, some authors have claimed that incorporation of preformed MRP into food systems [23] or the application of food processing practices to derive MRP within food [5] could result in improved oxidative stability. There have been several applications; addition of honey on turkey breast meat prior to heating [48], ground pork patties [49], tomato juice [50], sardine products [51], and soy sauce [52].

In the application of strategies for acrylamide and HMF mitigation in cookies, it should be considered the effect on potential health-promoting compounds. In addition, both aspects (risk and benefit) are concomitant during baking. This paper shows that AOA of water soluble compounds in cookies is deeply influenced by the variables related to both formulation (sugar, and leavening agent) and baking conditions which would also influence acrylamide and HMF formation as well. However, at this point a link between the reaction routes for HMF or acrylamide formation in cookies and levels of formed antioxidants during the baking process cannot be stated.

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