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Food Chemistry 97 (2006) 712–718
Food Chemistry
www.elsevier.com/locate/foodchem

In vitro DNA-protective activity of roasted wheat germ and fractions thereof

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Received 14 February 2005; received in revised form 17 May 2005; accepted 17 May 2005

Abstract

An ethanolic extract of roasted wheat germ was shown to scavenge free radicals, using the DPPH-test, and to protect DNA efficiently in vitro, using the 3D-assay. The DNA-protective activity of a coffee extract was comparatively lower and strongly dependent on the concentration applied. Fractionation of the wheat germ extract by preparative HPLC demonstrated that most of the DNA protecting properties were generated during the roasting process. Coupled GC–MS and HPLC–MS allowed identification of the main constituents of the active fractions. The contribution of genuine phenolic compounds was minor. Activity profiles of the radical-scavenging and of the 3D-assay test were not congruent. The attempt to extrapolate from in vitro measurements to the human in vivo situation is discussed.

Keywords: Roasted wheat germ; Coffee; 3D-assay; DNA repair; Radical scavenging

1. Introduction

Living organisms are continuously exposed to a number of endogenous and exogenous reactive oxygen/nitrogen species (ROS/RNS). The nature of this reactive species is either radical, such as superoxide anion radical, hydroperoxyl radical, lipid peroxyl radical and nitrogen dioxide, or non-radical, such as hydrogen peroxide, peroxynitrite, nitronium cation and singlet oxygen. Hydrogen peroxide and superoxides are the most abundant endogenous sources of ROS and occur as (side-)products of a number of oxidative processes in aerobic metabolism (normal aerobic respiration, β-oxidation, or as metabolites of stimulated leucocytes and macrophages). The exogenous sources consist of physical (ionising radiation, UV-A light) and chemical agents (tobacco smoke, pollutants, hyperoxic environment) (Alho & Leinonen, 1999). In living organisms all of them are potential precursors of the ultimate reactive oxygen species, the hydroxyl radical, which is capable of causing damage to proteins, lipids and nucleic acids. DNA damages may concern strand breaks, cross-links (to proteins, lipids), base/sugar alterations, and formation of DNA-adducts. Much circumstantial evidence suggests that free-radical biochemistry must be considered to have major significance in mutagenesis, carcinogenesis, ageing, and neurogenerative diseases, such as Parkinsons and Alzheimers (Haliwell & Gutteridge, 1990; Salles, Sattler, Bozzato, & Calsou, 1999). To protect itself against the deleterious effects of free radicals, the human body has evolved an antioxidant defence system that comprises enzymatic, metal chelating, and free radical-scavenging activities. In addition to endogenous defence, consumption of dietary antioxidants appears to be important (Ames, Shigenaga, & Hagen, 1993;

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doi:10.1016/j.foodchem.2005.05.050

Halliwell, 1999a). Exogenous antioxidants, especially free radical scavengers, have attracted especial interest, because they might protect the human body from free radicals (Saint-Cricq de Gaulejac, Provost, & Vivas, 1999). Therefore, rapid tests, based on radical (ABTS, DPPH) scavenging, have gained popularity. In vivo antioxidative, antimutagenic, or even anticarcinogenic properties were reasoned from antiradical properties shown in vitro. A novel DNA-protection assay (DNA Damage Detection, 3D-assay) was introduced by Salles et al. (1999). Plasmid or genomic DNA is treated with genotoxic agents in the presence or absence of the presumed antioxidants. All types of substrate DNA lesions can be detected after damage excision repair in microplate wells with chemiluminescence detection.

In previous work, an antioxidative ethanolic extract (AOE) of roasted wheat germ (Krings, El-Saharty, El-Zeany, Pabel, & Berger, 2000) was compared to extracts from other roasted foods. The concentration of conjugated diene hydroperoxides of tocopherol-free corn oil was used as an indirect method, and scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals served as a direct method (Krings & Berger, 2001). While the ethanolic extract from roasted wheat germ was more active than those of most other roasted foods, coffee extract was superior. In the present investigation, the DNA protection capability of wheat germ extract and fractions thereof was evaluated using the 3D-assay and compared to results obtained with the DPPH-radical scavenging test. The potential health-promoting impact that can be derived from the different in vitro measurements is discussed.

2. Materials and methods

2.1. Chemicals

Untreated wheat germ was kindly supplied by Bruno Zimmer, Obertal, Germany; and coffee (Melitta Cafe, Auslese mild), was purchased from the local market. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich, Deisenhofen, Germany. All solvents were p.a. grade and re-distilled prior to use.

2.2. Roasting of wheat germ

Twenty-five g samples were filled into centrifuge tubes (34 mm i.d. × 100 mm) sealed with screw caps (Teflon septum) and heated at 160 °C (oven temperature) for 20 min in a drying oven. The roasted samples were shock-cooled with liquid nitrogen and submitted to ethanolic extraction (Krings et al., 2000). Coffee, a popular roasted product, and also fresh wheat germ were submitted directly to extraction without any pretreatment.

2.3. Ethanolic extraction of wheat germ samples

Immediately after cooling, roasted germ samples, as well as untreated wheat germ and coffee, were stirred with 200 ml of ethanol for 16 h in glass-stoppered 300 ml Erlenmeyer flasks. After filtration, the extracts were concentrated to 20 ml under vacuum at 35 °C and stored for 24 h or longer at −20 °C in the dark; no loss of activity was observed (Krings et al., 2000). Such a concentrate was called an AOE (**a**nti**o**xidative **e**xtract). The reproducibility of the roasting and extraction process was controlled using the DPPH test (<10% SD; $n = 4$ for the radical-scavenging capacity).

2.4. Fractionation of wheat germ extracts

Separation was performed using a high performance liquid chromatography apparatus, consisting of two preparative pumps (Jasco PU-980 Intelligent HPLC Pump), a high pressure mixing chamber, a degasser (Shodex Degas), and a preparative diol column (Lichrosorb, 250 × 25 mm, 7 μm particle size, Merck) connected with a UV-detector (Jasco UV-1590). The detection wavelength was 280 nm. Samples were injected via a 2 ml injection loop (Rheodyne 7125). Solvents used were *n*-hexane (A) (Roti-SOLV, Roth) and ethanol (B) (absolute, AppliChem). The gradient was as follows: 10 min 100% A, from 10 to 70 min, linear gradient to 100% B, from 70 to 90 min 100% ethanol and finally back to 100% *n*-hexane within 10 min. The flow rate was adjusted to 10 ml min^{-1}. Twenty ml fractions were sampled every 2 min using a fraction collector (SF-3120, Advantec MFC Inc.). Fractions were re-concentrated to the injection volume of 2.0 ml by means of vacuum distillation, thereby removing the *n*-hexane almost completely. For GC- and LC–MS analysis, the most active fractions were pooled (range I: 8–24 min; II: 50–64 min; III: 66–74 min; IV: 76–84 min) and re-concentrated to 2 ml. Prior to silylation, aliquots of the fractions were evaporated to dryness and re-dissolved in pentane/diethyl ether (1:1.12, v.v.).

2.5. Radical-scavenging effects of fractions

The effect of each fraction of the AOE extracts of wheat germ and roasted wheat germ on DPPH-radicals was estimated according to Hatono, Kagawa, Yasuhara, and Okuda (1988) with some modifications. Fifty μl of each fraction/standard solution was added to 1 ml of a solution of DPPH radicals in ethanol and made up with ethanol to a final volume of 3 ml (final concentration of DPPH was 0.1 mM). The mixture was shaken vigorously and allowed to stand for 30 min; the absorbance of the resulting solution was measured at 517 nm, and activity was expressed as $-\Delta$ absorbance or as a percentage of the maximal de-coloration

(100%) of the stable DPPH radical. The concentration of total phenols in ethanolic extracts was determined by using the Folin–Ciocalteau reagent, as described elsewhere (Krings & Berger, 2001).

2.6. 3D-assay

The 3D-assay was from SFRI Laboratoire, Berganton, 33127 Saint Jean D'illac, France. The test was carried out according to the supplier's manual and Saint-Cricq de Gaulejac et al. (1999). In brief: 50 μl at a concentration of 1 μg ml^{-1}, ultra-purified plasmid DNA was applied to pre-washed and sensitised microtitre wells and gently stirred for 30 min at 30 °C, resulting in the adsorption of ~40 ng DNA per well. After repeated washing, 50 μl of each diluted sample (total extract: 1:10, 1:50, 1:250, and 1:1250 in water; each concentrated HPLC-fraction: 1:10 in water) or ultra pure (UHP) water was dispensed into a well. For the antiradical test, 25 μl of H_2O_2 (4 mM) and $FeCl_2$ (2 μM), respectively, were added to undamaged DNA in the presence or absence of the sample to be tested and incubated for 30 min at 30 °C with gentle shaking. Positive repair control, consisting of pre-damaged (UVC) DNA, was carried out with either UHP water (integrity of the test kid) or with diluted samples (unspecific inhibition of the repair reaction). After repeated washing, the incubation (3 h at 30 °C without shaking) with protein extracts (50 μl) allowed the excision of the lesion and repair synthesis in the presence of biotin-dUTP. The recognition of incorporated biotin-dUMP was achieved by ExtrAvidin coupled to horse-radish peroxidase and subsequent quantification of the light emitted after luminol addition by means of a luminometer (MXL Microplate luminometer, Dynex Hybaid Labsystems, Frankfurt, Germany). Each experiment was carried out at least in duplicate (SD < 12%). The DNA protection was expressed as the percentage reduction of the luminescence signal, whereas the signal of the unprotected DNA (maximal damage, maximal incorporation of dUTP) is defined as 100%.

2.7. GC- and LC–MS analyses of the AOE of roasted wheat germ

Main constituents were identified by means of high resolution GC–MS (HRGC–MS) and comparison of mass spectra and retention indices with those of commercial data bases (NIST, Wiley) using an HP 5890 Series II gas chromatograph coupled to a HP quadrupole mass spectrometer 5989A (interface: 250 °C, ion source: 250 °C, quadrupole: 100 °C, EI (70 eV), scan range *m/z* 33–500). The system was equipped with a DB 5 capillary column (30 m × 0.32 mm i.d. × 0.4 μm film thickness). Chromatographic conditions were: 1 μl injection volume, cool on-column injection, temperature programme 50 °C (3 min) and then to 280 °C (10 min) with a rate of 5 °C min^{-1}.

The structures of less or non-volatile compounds were derived from the interpretation of the GC–mass spectra of the corresponding trimethylsilyl derivatives. Silylation of 80 μl of sample was carried out using 20 μl of bis-(trimethylsilyl-)-trifluoro acetamide with 1% trimethyl-silyl chloride (Sigma) or by means of HPLC-MS.

LC–MS was carried out using a Shimadzu LC–MS-QP8000α (APCI-positive and negative mode) system, consisting of a Shimadzu DGU-12A degasser, a LC-10AD/LC-10ATVP gradient former and SPD-10AvP UV-detector (210 nm). Twenty μl of filtered (0.45 μm cellulose acetate) sample were injected on to a LiChrosphere 100 Diol 5 μm 250 × 25 mm, column (Merck) with a pre-column of the same material. Gradient elution was carried out with *n*-hexane (A) and ethanol (B) with a flow rate of 0.5 ml^{-1}. The gradient was as follows: 10 min 40% A, from 10 to 20 min, linear gradient to 100% B, from 20 to 25 min 100% B and finally back to 40% A within 10 min.

3. Results and discussion

3.1. DNA protection of the total ethanolic extracts

Prior work, on solvent extracts of roasted wheat germ, demonstrated strong antioxidative activity. The stability of tocopherol-free corn oil was improved with elevated roasting temperatures, indicating the involvement of Maillard-type antioxidants (Krings et al., 2000). A comparison of antioxidative properties of ethanolic extracts obtained from several roasted cereals using different methods resulted in some contradictions about their capability to protect lipids against autoxidation, and to scavenge the stable DPPH radical (Krings & Berger, 2001). The question of the potential physiological activity of the ethanolic extracts of roasted wheat germ, hazelnut, sweet almond and coffee remained unanswered.

Using the 3D-assay according to Saint-Cricq de Gaulejac et al. (1999) an exponential dose–response curve of repair activity of extracts of coffee and roasted wheat germ was obtained by incubation of DNA adsorbed in sensitised wells with $H_2O_2/FeCl_2$ (Table 1). Control experiments with pre-damaged DNA confirmed that the test kit was working properly. Ethanolic extracts of roasted hazelnut and sweet almond were also submitted to the 3D-assay, but yielded a strong unspecific repression of the repair reaction (data not shown). The ethanolic extract of roasted wheat germ exhibited a clear correlation between the concentration and DNA protection, and apparently this correlation was not linear. With the coffee extract, the situation was

Table 1
DNA protection by ethanolic extracts of roasted wheat germ and coffee

	Non-specific repression of repair reaction	DNA damage (genotoxicity)	DNA protection (%)
Wheat germ			
1:10	None[a]	n.d.*	70
1:50		n.d.	65
1:250		n.d.	51
1:1250		n.d.	32
Coffee			
1:10	None[a]	Yes[#]	55
1:50		Yes	0
1:250		Yes	−20
1:1250		Yes	−10

Compared to the control *no significant/[#]an increased luminescence signal was detected without addition of Fenton's reagent.
[a] Tested for the highest concentration only.

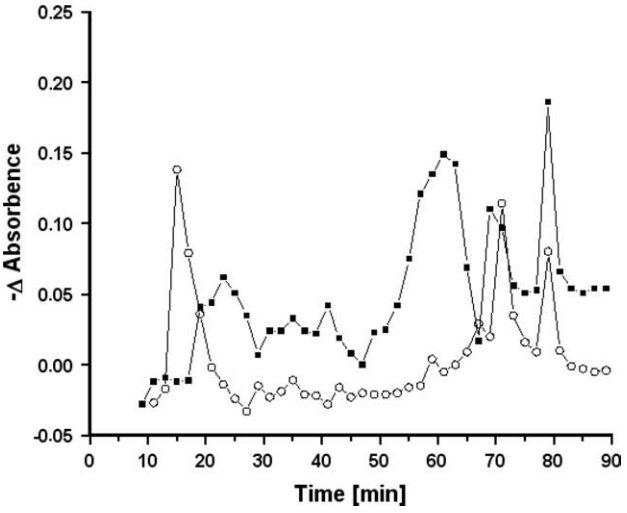

Fig. 1. DPPH radical-scavenging properties of prep. HPLC fractions: ■, AOE of roasted wheat germ; ○, AOE of fresh wheat germ.

Table 2
Total phenol concentration of the AOE of roasted wheat germ

Sample	Total phenol concentration (μg ml^{-1})
Total extract	29.0
Range I	8.0
Range II	1.0
Range III	9.0
Range IV	1.5

more complex: the highest concentration also had DNA-protective activity which dramatically decreased with the first 1:5 dilution step and turned then into the opposite effect on further dilution. This DNA damaging capability was confirmed by the control experiment (no Fenton reagent applied), in which the coffee extracts caused a significant luminescence signal in all dilutions (compared to untreated intact DNA), whereas dilutions of the AOE of roasted wheat germ did not result in any DNA damage. The apparent DNA protection of the 1:10 dilution of the coffee extract could be explained by the repression of the Fenton reaction (generation of the OH radical), either by chelating of the iron ions or by oxidation of Fe^{2+} to Fe^{3+}. The same finding was reported by Stadler, Turesky, Mueller, Markovic, and Leong-Morgenthaler (1994) who found that coffee accelerated the oxidation of 2′-deoxyguanine (2′-dG) to 8-OH-2′dG at low dosages. However, the formation of 8-OH-2′dG was decreased by increasing the dosage of coffee, which the authors attributed to interactions of the highly electrophilic hydroxyl radical with coffee constituents (Stadler et al., 1994; Turesky, Stadler, & Leong-Morgenthaler, 1993).

3.2. Fractionation of the ethanolic extract of wheat germ extracts

To obtain a more detailed picture of the nature of antiradical and DNA-protecting capabilities, the ethanolic extract of roasted wheat germ was submitted to preparative HPLC fractionation on a diol phase. A comparison of the radical scavenging properties of roasted and untreated wheat germ is shown in Fig. 1. Previous work demonstrated that the ethanolic extract of fresh wheat germ offered antiradical and antioxidative properties, but this was less pronounced than after roasting (Krings et al., 2000). For both extracts, a qualitatively similar activity pattern was found in the last third of the chromatogram (in the more polar fractions). However, the last activity peak was significantly more intensive for the AOE of the roasted sample. UV detection at 280 nm, LC–MS measurements, and an increased total phenol content gave evidence for the presence of phenolics (Table 2). A statistically significant correlation between the total phenolics and the antioxidant activity was found for fruits, vegetables and grain products (Velioglu, Mazza, Gao, & Oomah, 1998). The semi-polar fractions (range II) showed a strong activity for the roasted sample only, whereas a sole sharp activity maximum was observed in the non-polar, range I, of the untreated extract. This activity can be attributed mainly to tocopherols which are mostly degraded during the heating process (Krings et al., 2000). In conclusion, the roasting process generated an excess of radical scavenging properties in the semipolar (new activity) and polar (increased activity) ranges, while the tocopherols are lost.

3.3. DNA protection properties of fractions

The DPPH radical-scavenging activity pattern of each fraction of the AOE of roasted wheat germ was compared to its DNA protection activity (Fig. 2). The overall activity patterns were similar, but there were significant differences, too. The very first two and some later-eluting fractions (22–24; 26–28; 30–34; 38–40 min)

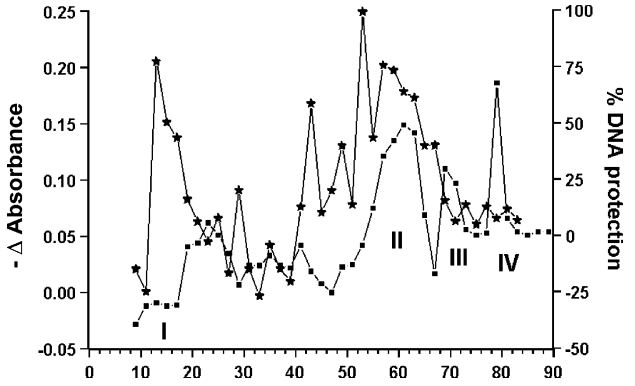

Fig. 2. Bioactivity of HPLC fractions of the AOE from roasted wheat germ: ■, DPPH-scavenging properties; ★, effect on DNA; positive values indicate protection, negative values damage.

were classified as genotoxic by the 3D-assay, because the luminescence signal (extent of DNA damage) exceeded the value obtained with the control (water added instead of sample). The control experiment for genotoxicity was not carried out for each fraction but, as was demonstrated for the experiment using coffee extract, the 3D-assay indicated genotoxic constituents by luminescence larger than in the control experiment (Table 1). The main differences in the activity curves were found in ranges I and IV. Range I gave strong DNA protection which was not accompanied by any DPPH radical-scavenging activity. The inverse situation was found with range IV: a sharp maximum of DPPH radical-scavenging did not concur with DNA protection.

3.4. Chemical composition of protective fractions

A tentative elucidation of the major chemical composition of pooled active fractions was carried out by means of coupled GC- and HPLC–mass spectrometry. Several compounds or chemical classes of substances were identified in the four ranges. Many of these substances were also present in native wheat germ, but some occurred during roasting, for example sugar alcohols, oxo-pentoses, oxo-hexoses and oxo-sugars, which are typical reaction products in cooked and roasted foods. Myo-inositol and phosphoric acid are products of hydrolysis of the well known antioxidative wheat constituent phytin (Empson, Labuza, & Graf, 1999).

The major constituents of the DNA-protecting range I were found to be mono- and diacylglycerols, as well as phytosterols. Although no DPPH-scavenging activity was observed, DNA was efficiently protected against damage induced by the Fenton reaction. A non-specific inhibition of the 3D-assay can be ruled out (Table 1). The chelating of Fe^{2+}-ions and, therefore, suppression of the OH-radical generation by the Fenton reaction is more likely than an OH-radical-scavenging activity. This is supported by the absence of DPPH scavenging activity in this range. In the semipolar ranges II and III, the two activity curves of Fig. 2 run almost parallel. The major constituents identified in these fractions were found to lack noticeable DPPH-scavenging properties when tested as single components (5 μM) and compared to α-tocopherol (Table 3).

Table 3
DPPH-scavenging properties of some compounds compared to α-tocopherol (5 mM)

Compound	Scavenging capacity[a] (%)
α-Tocopherol	84
Glucose	3.5
Sorbitol	2.5
Sucrose	1.0
Gluconic acid	1.5
Glucono-δ-lactone	5.0

[a] Maximal de-coloration was set to 100%.

The DPPH-scavenging and the DNA-protecting activity of range II was generated by the roasting process and was of non-phenolic nature (see Fig. 1 and Table 2). It is known that Maillard pathways can yield low-molecular weight antioxidants, such as reductones and aminoreductones (Pischetsrieder, Schoetter, & Severin, 1998; Singhara, Macku, & Shibamoto, 1998) but, under the conditions in which the ethanolic AOE of roasted wheat germ was prepared, not in GC–MS detectable amounts. Tressl, Wondrak, Kersten, Kriiger, and Rewicki (1998) identified and characterised Maillard-type polymers with antioxidative activity by FAB-MALDI-TOF-MS and the DPPH reduction assay (Tressl et al., 1998). More recently, the antioxidant potential of ethanol-extractable melanoidins from rye bread crust was evaluated and pronyl-lysine, a carbohydrate modification of protein-bound lysine side chains, was identified as an active principle which was not present in the ingredients prior to the baking process (Lindenmeier, Faist, & Hofmann, 2002). Using wheat gluten in the presence of carbohydrate sources as a model system, the formation of protein-bound pyrrolinone reductones was proven. These findings suggest that the antioxidative principles of roasted wheat germ formed during the heating process are not the classical low-molecular weight (mainly phenolic) antioxidants.

In the most polar fractions, range IV, a sharp maximum of DPPH radical-scavenging occurred, but no DNA protection was recorded. The kinetics of the DPPH-scavenging reaction, however, were remarkably slow. From preparative HPLC chromatograms with UV-detection at 280 nm and HPLC–MS data (not shown), and from the total phenol content (Table 2), phenolic constituents are expected to be the active principles. Phenols are well known to react with the stable DPPH radical, but reaction kinetics decrease significantly with the molecular mass. Therefore, the highly reactive OH-radical might not have been inactivated efficiently, before it encountered a DNA molecule.

4. Conclusions

The ethanolic extract of roasted wheat was shown to scavenge free radicals (DPPH-test) and, using the 3D-assay, to protect DNA efficiently in vitro. Fractionation of the extract showed that, during the roasting process, DNA-protecting properties were generated. But does the extract offer the same DNA protecting properties in vivo? When the OH radical is formed near the DNA, it will attack the DNA bases immediately. As far as is known, no other free radical species generates such a wide variety of end-products in DNA (Halliwell, 1999b). The 3D-assay is able to detect all kinds of DNA lesions, and especially lesions induced by active oxygen species or free radicals. Therefore, a bio-analytical system to evaluate DNA-protection properties, consisting of OH radical generation by the Fenton reaction in the presence of genomic DNA (and the antioxidant to be tested), appears to be close to in vivo conditions. Hence, the ethanolic AOE of roasted wheat might act as a protectant against radical attacks on DNA in vivo, if the active constituents of the AOE ever reached the target compartment in the cells at an appropriate concentration. To this end, the extract, or parts thereof, must remain active when passing the digestive track. Bio-availability, absorption, metabolism and pharmaco-kinetics must be considered before attempting to extrapolate from in vitro procedures to the human in vivo situation (Moure et al., 2001).

It is common knowledge that the human health status is improved by a preference for plant foods. Natural antioxidants from dietary plants are reported to prevent oxidative damage by free radicals and active oxygen, and to decrease the occurrence of cardiovascular diseases and cancer, and retard ageing (Hirose, Imaida, Tamano, & Ito, 1994). Individuals who eat plenty of fruits and vegetables tend to have higher plasma and tissue levels of antioxidants. However, the conclusion that an elevated supply of ascorbate, tocopherols, and β-carotene was protective is not stringent. It is merely conclusive that a diet that achieves elevated plasma levels of natural antioxidants is protective (Halliwell, 1999b). This differentiated view is supported by a clinical study, where the 8-OHdG levels in urine were not decreased by an increased supply of vitamin C, E or β-carotene, but only when vegetables, such as Brussel sprouts, were consumed by volunteers (Halliwell, 1999a). Another important feature of the antioxidant network in the living organism is that its components act in synergy to destroy activated oxygen species. The influence of exogenous antioxidants on this usually balanced system cannot be evaluated by in vitro experiments (Chaudière & Ferrari-Iliou, 1999). The antioxidant defence in organisms consists, not only of low-molecular weight compounds, but also of protective enzymes and repair systems. The enzymatic defence systems can be induced with different stimulants. In consequence, even genotoxic compounds may, in low concentrations, improve the antioxidative status in the body by inducing the defence system. Antioxidant inhibitors of lipid peroxidation or scavengers of the stable DPPH radical may not protect other targets, such as DNA and proteins, against damage and sometimes may even aggravate such damage (Halliwell, 1999a).

Acknowledgements

The authors are grateful to the Fonds der Chemischen Industrie, Frankfurt, to Bruno Zimmer GmbH, to Y.S.I. El-Saharty, and to C. Theobald for scientific and financial support.

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