

Prooxidant Capacity of Thermoxidised Plant Oils

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Abstract

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The prooxidant capacity of rapeseed, sunflower, soybean, and olive oil was determined before and after heating at a temperature of 180°C for 2, 4, and 6 hours. It was quantified as losses of α -tocopherol caused by the studied oils during 24-h incubation of their acetone–methanol solutions with addition of α -tocopherol at 30°C, whereas the decrease in α -tocopherol concentration was studied as a decrease in antioxidant capacity determined by the spectrophotometric DPPH (2,2-diphenyl-1-picrylhydrazyl) method. During heating of all the studied plant oils, the prooxidant capacity grew due to the formation of reactive lipid oxidation products, but, except the sunflower oil, it did not depend on the time of heating – after the initiatory increase, the prooxidant capacity typically remained approximately constant or decreased. The prooxidant capacity of the heated oils ranged from 58 mg to 360 mg α -tocopherol/kg and decreased in the order soybean oil > rapeseed oil > olive oil $\approx$ sunflower oil. It did not correlate with the content of polymerised triacylglycerols (except the sunflower oil) and was generally higher than the residual content of α -tocopherol.

Keywords: antioxidant activity; frying; polymerised triacylglycerols; prooxidant activity; tocopherols

In spite of the low acute toxicity of oxidised fats and oils (ESTERBAUER 1993), a lot of undesirable effects were observed in some long-term tests with them (ALEXANDER 1981; IZAKI *et al.* 1984; KUBOW 1990; ESTERBAUER 1993; CHOW 2007; TOTANI *et al.* 2008; OMWAMBA *et al.* 2010). The symptoms can be related to a decreased digestibility and availability of oxidised lipids (ESTERBAUER 1993; OMWAMBA *et al.* 2010), an incorporation of modified fatty acids into membranes or tissues (ALEXANDER 1981; CHOW 2007) or an effect of modified fatty acids on the metabolism of essential fatty acids (ALEXANDER 1981). But probably the most important cause consists in an increased oxidative stress in the gastrointestinal tract or *in vivo* (OMWAMBA *et al.* 2010).

The lipid oxidation products formed in food are able to induce undesirable oxidation processes *in vivo* (UDILOVA *et al.* 2003; CHOW 2007; OMWAMBA *et al.* 2010). They can oxidise proteins (oxidisable amino acids such as cysteine or tyrosine) (GARD-

NER 1983; CHOW 2007), DNA (YANG & SCHAICH 1996; MARTINEZ *et al.* 2003), unsaturated fatty acids (UDILOVA *et al.* 2003; GOMES *et al.* 2008, 2011) and other compounds (see below). The effects of these reactions are similar to the oxidative damage due to free radicals and other oxidants originating in some cases *in vivo* or entering into the body for example as a result of smoking (WILLCOX *et al.* 2004). *In vivo* uncontrolled oxidation (i.e. an increased oxidative stress) can participate in pathological processes and illnesses such as cardiovascular, tumour or neurodegenerative diseases (WILLCOX *et al.* 2004; CHOW 2007). The oxidation of proteins, DNA or fatty acids can be a direct cause of these diseases (WILLCOX *et al.* 2004). However, *in vivo* oxidation of antioxidants (ascorbic acid, tocopherols and others) can also be important. For example, some lipid oxidation products are not able to oxidise DNA (STEENKEN & JOVANOVIĆ 1997), but these compounds can cause losses of antioxidants (REPORTER & HARRIS 1961;

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IZAKI *et al.* 1984; ESTERBAUER 1993; CHOW 2007), which are subsequently missing in DNA protection against reactive hydroxyl radicals arising in some cases *in vivo* (WILLCOX *et al.* 2004).

Some *in vivo* signs were reported as the results of oxidised lipid intake; they included an increased level of the compounds reacting with thiobarbituric acid in liver (IZAKI *et al.* 1984; ESTERBAUER 1993; CHOW 2007; OMWAMBA *et al.* 2010), an increased content of oxidised low density lipoproteins in blood plasma (ESTERBAUER 1993), a decreased content of tocopherols and other antioxidants in liver and serum (REPORTER & HARRIS 1961; ESTERBAUER 1993; IZAKI *et al.* 1984; CHOW 2007), oxidative damage to membranes, an increase of saturated fatty acid content in them or oxidative damage to proteins (CHOW 2007). *In vitro*, a prooxidant activity of oxidised fats and oils and/or lipid oxidation products was documented for example in the model systems created to study the oxidative changes of lipids (GOMES *et al.* 2008, 2011), proteins (POKORNÝ *et al.* 1992) or tocopherols (KAJIMOTO *et al.* 1990). However, there is a lack of information concerning the quantified prooxidant activity of oxidised (thermoxidised) fats and oils, i.e. their prooxidant capacity, especially in relation to other sources of oxidative stress and typical intake of antioxidants.

Therefore, this work was aimed at the quantification of prooxidant activity (capacity) of thermoxidised plant oils. For this purpose, the oxidative losses of

α -tocopherol caused by these oils were studied during 24-h incubation of the oil samples (as acetone–methanol solutions) with an addition of α -tocopherol at 30°C. To make the work more convenient and effective, the decrease in α -tocopherol concentration was followed as a decrease in antioxidant capacity, which can be determined using a quick spectrophotometric test. Subsequently, the prooxidant capacity of thermoxidised oils was compared with a content of polymerised triacylglycerols, antioxidant capacity, and residual content of tocopherols.

MATERIAL AND METHODS

Plant oils and their oxidation. The used vegetable oils were purchased in ordinary shops. The rapeseed, sunflower, and soybean oils were fully refined, while the olive oil was a commercial mixture of refined and virgin oils. The basic chemical characterisation of all oils is summarised in Table 1.

The oil samples (25 g $\pm$ 1%) were heated (oxidised) in beakers (volume 100 ml, external diameter 47 mm) on a heating plate (Präzitherm PZ 28-2; Gestigkeit GMBH, Düsseldorf, Germany) with a steel adapter with cylindrical holes (internal diameter 48 mm and depth 28 mm) for a better heat transfer. The temperature of the heating plate was set to 220 $\pm$ 1°C. Under these conditions, the temperature of the oil increased during 20 min and then fluctuated

Table 1. Chemical characterisation of used (non-heated) plant oils

	Olive oil	Sunflower oil	Soybean oil	Rapeseed oil
Fatty acids (%)				
Palmitic	13.5 $\pm$ 0.2	6.8 $\pm$ 0.1	11.0 $\pm$ 0.8	5.5 $\pm$ 0.7
Stearic	3.1 $\pm$ 0.1	3.5 $\pm$ 0.0	4.0 $\pm$ 0.1	1.7 $\pm$ 0.1
Other saturated	0.7 $\pm$ 0.0	1.4 $\pm$ 0.0	1.3 $\pm$ 0.1	1.1 $\pm$ 0.1
Oleic	67.9 $\pm$ 0.4	24.4 $\pm$ 0.0	22.8 $\pm$ 0.2	59.3 $\pm$ 0.6
Other monoenic*	3.7 $\pm$ 0.3	1.0 $\pm$ 0.0	1.6 $\pm$ 0.0	4.2 $\pm$ 0.2
Linoleic	10.4 $\pm$ 0.0	62.3 $\pm$ 0.1	51.4 $\pm$ 0.4	21.1 $\pm$ 0.0
α -Linolenic	0.5 $\pm$ 0.0	0.1 $\pm$ 0.0	6.7 $\pm$ 0.1	6.1 $\pm$ 0.1
Other polyunsaturated*	0.2 $\pm$ 0.0	0.5 $\pm$ 0.0	1.2 $\pm$ 0.0	1.0 $\pm$ 0.0
α -Tocopherol (mg/kg)	168 $\pm$ 1	580 $\pm$ 1	121 $\pm$ 5	271 $\pm$ 3
Sum of β - and γ -tocopherol (mg/kg)	12 $\pm$ 0	28 $\pm$ 1	816 $\pm$ 2	307 $\pm$ 2
δ -Tocopherol (mg/kg)	1 $\pm$ 0	22 $\pm$ 1	268 $\pm$ 1	8 $\pm$ 0
Peroxide value (meq./kg)	5.4 $\pm$ 0.1	2.6 $\pm$ 0.3	3.2 $\pm$ 0.1	1.7 $\pm$ 0.1

*including *trans*-isomers; results are expressed as mean $\pm$ standard deviation, $N = 2$ for fatty acids determination, resp. $N = 3$ for the other parameters

between 178°C and 182°C. The oil temperature was monitored in parallel experiments (beakers), which were not used for the following chemical analyses. The oil samples were heated for two, four, and six hours. For sampling, the whole beaker was always removed from the hot plate. Thus, the oil volume in the beakers was constant during the entire heating period. For all sets of conditions (i.e. for all oils and heating periods), three experiments were performed.

Determination of prooxidant capacity. The prooxidant capacity was quantified as a loss of α -tocopherol detected during 24-h incubation of oil samples (as acetone-methanol solutions) with addition of α -tocopherol at 30°C, whereas the decrease in α -tocopherol concentration was followed as a decrease in antioxidant capacity. For this purpose, the oil samples (approximately 0.25 g) were weighed to volumetric flasks ($V = 25$ ml) and dissolved in 10 ml of acetone. Then, 5 ml of methanol containing approximately 300 μ g of α -tocopherol was added and, after mixing, the closed volumetric flasks were incubated at 30°C for 24 h in the dark. After incubation, the antioxidant capacity of samples (the mixtures of oil samples with α -tocopherol) was determined (see below, starting from DPPH addition) and a decrease in the antioxidant capacity during incubation was ascertained. The antioxidant capacity of the mixtures of oil samples with α -tocopherol before incubation was calculated as the sum of the antioxidant capacity of oil samples (see later) and the known weight of α -tocopherol addition. The decrease in antioxidant capacity during incubation (in mg α -tocopherol/kg) was considered to be the prooxidant capacity, because no loss of α -tocopherol was detected during incubation of the α -tocopherol solution without oil sample.

Determination of antioxidant capacity. The antioxidant capacity was determined spectrophotometrically as the ability of analysed samples to inactivate free DPPH radicals (2,2-diphenyl-1-picrylhydrazyl; Sigma Aldrich, St. Louis, USA) (BRAND-WILLIAMS *et al.* 1995), under the following modified conditions: the oil samples (approximately 0.25 g) were weighed to volumetric flasks ($V = 25$ ml) and dissolved in 10 ml of acetone. Then, a methanol solution of DPPH radical (5 ml, $c = 0.2$ mg/ml) was added and the flasks were filled to the mark with methanol. After mixing, the closed volumetric flasks were incubated for 30 min at room temperature. Consequently, the values of absorbance at 518 nm were measured in 1-cm rectangular cells against pure methanol using a Cary 100 Bio spectrophotometer (Varian, Palo Alto, USA). In the case of samples with a higher

content of polymerised triacylglycerols, which did not provide pellucid final solutions susceptible to correct absorbance measurement, the solutions were filtered before absorbance measurement using Nylon syringe filters with pore size 0.45 μ m (Carl Roth, Karlsruhe, Germany). The antioxidant capacity was expressed in mg α -tocopherol/kg using the calibration straight line in which the oil samples were replaced by methanol solutions of α -tocopherol containing from 0 μ g to 500 μ g of α -tocopherol.

Determination of polymerised triacylglycerols. The polymerised triacylglycerols (TAG) were determined using HP-SEC with refractometric detection (RÉBLOVÁ 1998). The system consisted of an LCP 4000.11 high-pressure pump (Ecom, Prague, Czech Republic), HP 1050 series autosampler and HP 1047A series refractometric detector (Agilent Technologies, St. Clara, USA). The chromatographic separation was performed using a PL gel MIXED-E SEC column (7.5 mm $\times$ 300 mm, 3 μ m) equipped with a guard column (7.5 mm $\times$ 50 mm, 5 μ m; Agilent Technologies). Tetrahydrofuran was used as the mobile phase at a flow rate of 0.6 ml/minute. The oil samples were prepared for the analysis by dissolution in tetrahydrofuran (approximately 60 mg/ml) and 5 μ l of the solutions were injected into the column. The percentage of polymerised TAG was quantified using the area-normalisation method.

Determination of tocopherols. The tocopherols were determined using reverse-phase HPLC with amperometric detection (TROJÁKOVÁ 2001). The system consisted of an LCP 4020.31 non-steel high-pressure pump (Ecom, Prague, Czech Republic), Rheodyne 7725i manual sample injector (Rheodyne, Oak Harbor, USA), LCO 101 column heater set to 28°C (Ecom, Prague, Czech Republic) and HP 1049A series amperometric detector equipped with a glassy-carbon working and solid state Ag/AgCl reference electrode (Agilent Technologies, St. Clara, USA). The chromatographic separation was performed using a Hypersil ODS column (4.6 mm $\times$ 200 mm, 5 μ m; Agilent Technologies, St. Clara, USA). A mixture of acetonitrile and methanol (1 : 1, v/v) containing LiClO₄ (0.02 mol/l) and NaCl (0.005 mol/l) was used as the mobile phase at a flow rate of 1.0 ml/minute. The oil samples were dissolved in acetone (approximately 0.1 g/ml) and 20 μ l of the solutions were injected into the column. The detection potential was set to 1.05 V. The contents of individual tocopherols were quantified using the corresponding calibration lines.

Determination of fatty acid composition. The analysis of fatty acid composition was done by GC

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after derivatisation using a base-catalysed reaction with KOH-methanol as the reagent and extraction with hexane (NOLLET 1996). The GC analysis was carried out on an Agilent Technologies 6890N gas chromatograph (Agilent Technologies, Palo Alto, USA) equipped with a flame ionisation detector and Supelco SP 2560 capillary column (100 × 0.25 mm *i.d.*, thickness of 0.2 µm; Supelco, Bellefonte, USA). The injector was held at 240°C (split, 75 : 1), the column temperature was programmed from 175°C to 240°C at the rate of 4°C/minute. Helium, at the flow rate of 0.8 ml/min, was used as the carrier gas, 1 µl sample was injected. The results are expressed in relative percentages of each fatty acid and calculated by the internal normalisation method using the chromatographic peak areas.

Other analytical methods. Peroxide value was determined using the IUPAC titrimetric method (IUPAC 1964).

Result processing. Differences between samples were analysed by applying Student's *t*-test at the probability level of 0.05. The existence of a relation between the prooxidant capacity and the content of polymerised triacylglycerols was tested using the Pearson correlation coefficient.

RESULTS AND DISCUSSION

Prooxidant capacity of heated plant oils. The prooxidant capacity of all analysed samples is summarised in Table 2. Not only the heated plant oils, but also the unheated ones showed a certain prooxidant activity toward α -tocopherol, although only the prooxidant capacity of the unheated sunflower oil was significantly different from zero ($\alpha < 0.05$). This fact, i.e. the detected prooxidant activity of the unheated plant oils, agrees with nonzero peroxide values of the used unheated

oils (Table 1), because hydroperoxides exhibit significant oxidative (prooxidative) ability, not only against tocopherols (GARDNER 1983; YANG & SCHAICH 1996; MARTINEZ *et al.* 2003; UDILOVA *et al.* 2003).

During heating of all the studied plant oils, the prooxidant capacity grew due to the formation of reactive lipid oxidation products, i.e. oxidative agents (CHOW 2007; OMWAMBA *et al.* 2010), although the ascertained growth was not significant in all cases ($\alpha < 0.05$). With the exception of sunflower oil, the prooxidant capacity of oils did not increase in a monotonous way – it increased considerably at the beginning and then remained approximately constant or even decreased. These results agree with results obtained in experiments with animals in which the overall mortality rate and incidence of tumours and other pathological changes or disease conditions, which can have connection with the prooxidant activity of heated plant oils (UDILOVA *et al.* 2003; OMWAMBA *et al.* 2010), were not always correlated with the extent of oil heating (CHOW 2007). This is logical considering the sequence of chemical transformations of lipids in the course of oil heating. Reactive hydroperoxides and aldehydes, which are probably the dominant lipid oxidation products showing a prooxidant activity (ESTERBAUER 1993; YANG & SCHAICH 1996; UDILOVA *et al.* 2003), are transformed to other lipid oxidation products in later stages of oxidation (CHOE & MIN 2007) and the aldehydes can escape from heated oil via volatilisation (SEPANEN & CSALLANY 2002). So, the prooxidant capacity of the heated (thermoxidised) oils depended mainly on the type of oil and decreased in the order soybean oil > rapeseed oil > olive oil $\approx$ sunflower oil, probably with respect to different content of linolenic acid (Table 1) providing higher amounts of reactive aldehydes (FULLANA *et al.* 2004).

The prooxidant capacity of the heated (thermoxidised) oils ranged approximately from 60 mg to 360 mg

Table 2. Changes of prooxidant capacity (in mg α -tocopherol/kg) of plant oils during heating at temperature 180°C

Oil	Time of heating (h)			
	0	2	4	6
Olive	19 ± 13 ^{aA*}	78 ± 10 ^{bA}	71 ± 13 ^{bA}	58 ± 2 ^{bA}
Sunflower	47 ± 10 ^{aA}	58 ± 4 ^{aA}	63 ± 11 ^{aA}	77 ± 15 ^{aA}
Soybean	50 ± 26 ^{aA*}	360 ± 5 ^{dC}	301 ± 23 ^{cC}	187 ± 12 ^{bC}
Rapeseed	61 ± 48 ^{aA*}	150 ± 2 ^{aB}	141 ± 22 ^{aB}	135 ± 10 ^{aB}

Data are expressed as mean ± standard deviation for three experiments; *value is not significantly different from zero ($\alpha = 0.05$); ^{a-d}values with the same symbol in the same row are not significantly different ($\alpha = 0.05$); ^{A-C}values with the same symbol in the same column are not significantly different ($\alpha = 0.05$)

α -tocopherol/kg. This means that a medium serving of French fries containing typically 18 g of thermoxidised oil (Agricultural Research Service 2013) could initiate an oxidative loss of α -tocopherol ranging from 1 mg to 6.5 mg. It could be considered as a negligible amount in comparison with other sources of oxidative stress (WILLCOX *et al.* 2004; BÓKKON *et al.* 2010). However, providing selective reactions of the lipid oxidation products with lipophilic tocopherols, and above all with the most reactive α -tocopherol (KAMAL-ELDIN & APPELQVIST 1996), the discussed serving of French fries could cause *in vivo* and/or in the gastrointestinal tract (HALLIWELL *et al.* 2000) a loss of α -tocopherol ranging approximately from 6% to 40% of the daily vitamin E recommended dietary allowance for adults (EITENMILLER 2004). Though these values can be in part compensated by tocopherols (α -tocopherol) contained in the consumed French fries (see below), they are quite alarming, especially with respect to frequently insufficient vitamin E intake (MARAS *et al.* 2004) and high consumption of fried foods in the groups of children and adolescents (ENNS *et al.* 2003; RANGAN *et al.* 2011).

Comparison of prooxidant capacity with other parameters. The relation between the prooxidant capacity and the content of polymerised triacylglycerols is shown in Figure 1. There is not any correlation between these two parameters, neither for all oils together nor for individual oils separately except the sunflower oil ($\alpha < 0.05$). Therefore, the health risks of fats and oils after frying cannot be generally derived from the content of polymerised triacylglycerols, which is normally considered as one of the main parameters suitable to follow the chemical reactions taking place in fats and oils during frying (GERTZ &

MATTHÄUS 2012). This is logical, the polymerised triacylglycerols represent the final products of oil thermal treatment (CHOE & MIN 2007), whereas the prooxidant activity of thermoxidised fats and oils is probably caused mainly by hydroperoxides and aldehydes (ESTERBAUER 1993; YANG & SCHAICH 1996; UDILOVA *et al.* 2003), i.e. the intermediate products of lipid oxidation (CHOE & MIN 2007).

The comparison between the prooxidant capacity and other parameters, i.e. antioxidant capacity, content of α -tocopherol and total content of tocopherols, is shown in Figure 2. Although the studied oils manifested a prooxidant activity to quite easily oxidisable α -tocopherol (KAMAL-ELDIN & APPELQVIST 1996), they also showed an antioxidant activity to reactive free radicals (DPPH free radicals in this case; Figure 2A), alike as it was observed in the case of coffee beverages for example (LÓPEZ-GALILEA *et al.* 2007). The antioxidant activity, induced by tocopherols above all (see below), some other phenolic compounds [in olive oil mainly (VISIOLI *et al.* 2002)] and/or some non-enzymatic browning reaction products arising during lipid oxidation (MANZOCCO *et al.* 2000), could eliminate or decrease the prooxidant activity of lipid oxidation products. However, while the antioxidant capacity was approximately fifteen to thirty times higher than the prooxidant capacity in the unheated oils, the antioxidant capacity dominated over the prooxidant capacity only by factor two to seven in the case of heated oils. It was caused by an increase in the prooxidant capacity during thermoxidation of plant oils (see above) as well as by a fall of the antioxidant activity during the same process (caused mainly by losses of tocopherols – see below).

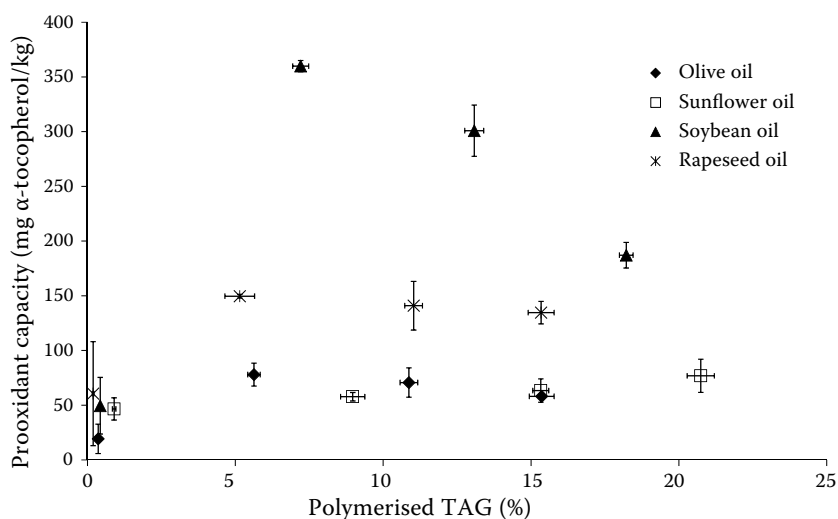


Figure 1. Relation between prooxidant capacity and content of polymerised triacylglycerols during heating of plant oils at temperature 180°C

Data are expressed as mean $\pm$ standard deviation for three experiments

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An analogous confrontation was also obtained for the content of α -tocopherol (Figure 2B) and the total content of tocopherols (Figure 2C). The total content of tocopherols was from nine to twenty-five times higher than the prooxidant capacity in the unheated oils, but at the most five times higher than the prooxidant capacity in the heated oils. Furthermore, the prooxidant capacity was higher than the total content of tocopherols in the case of heated olive oil and in sunflower and rapeseed oils heated for a long time. For α -tocopherol, the most easily oxidisable tocopherol (KAMAL-ELDIN & APPELQVIST 1996), the differences between unheated and heated oils were even more distinct. The content

of α -tocopherol was from two to twelve times higher than the prooxidant capacity in the unheated oils, but it was only in one case higher than the prooxidant capacity in the heated oils. It means that the studied thermoxidised oils typically had a negative balance of α -tocopherol and often also of total tocopherols; i.e. the losses of tocopherols (α -tocopherol), which could be initiated *in vivo* and/or in the gastrointestinal tract by these oils, are higher than the content of tocopherols (α -tocopherol) in these oils. In the case of α -tocopherol, which is the *in vivo* most active form of vitamin E (EITENMILLER 2004) and also the most reactive tocopherol (KAMAL-ELDIN & APPELQVIST 1996), the above discussed medium

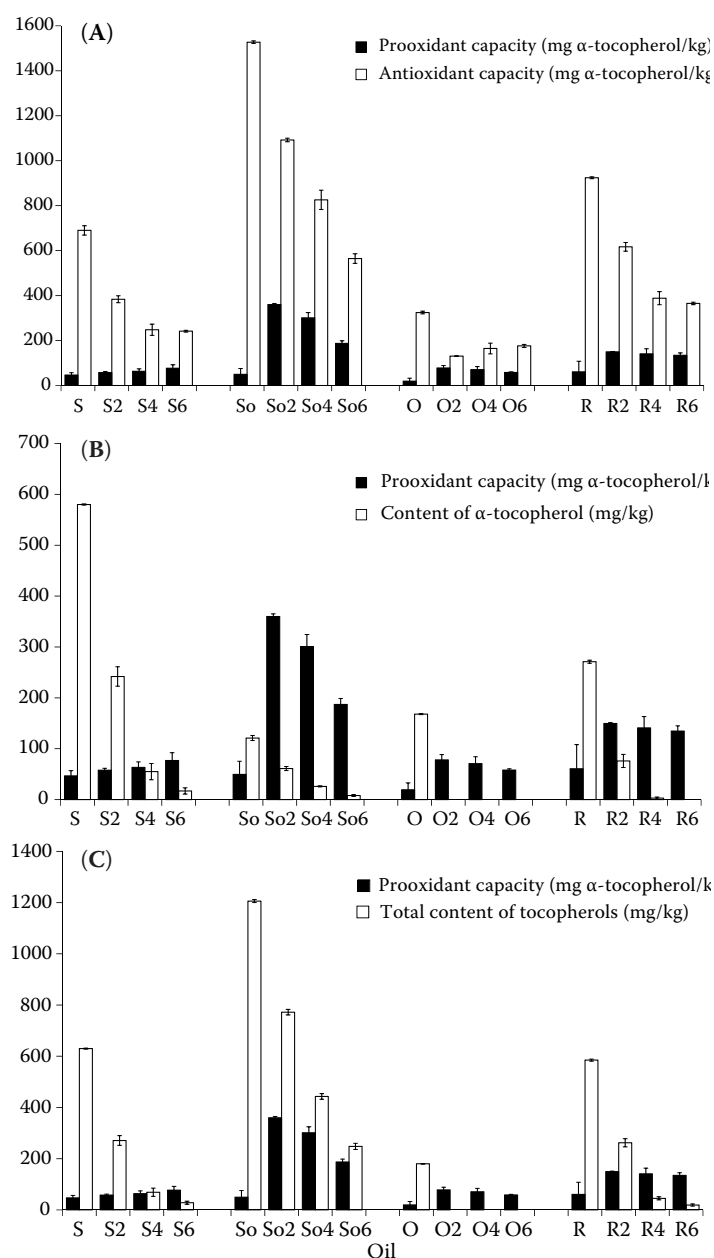


Figure 2. Comparison of prooxidant capacity with antioxidant capacity (A), content of α -tocopherol (B), and total content of tocopherols (C) during heating of plant oils at temperature 180°C

Data are expressed as mean $\pm$ standard deviation for three experiments; S – non-heated sunflower oil; So – non-heated soybean oil, O – non-heated olive oil; R – non-heated rapeseed oil; S2 – sunflower oil after two-hour heating etc.

serving of French fries containing 18 g of the studied thermoxidised oils could be a source of at the most 3 mg of utilisable α -tocopherol (i.e. the content of α -tocopherol in this serving is reduced by the respective prooxidant capacity). It represents approximately 20% of the daily vitamin E recommended dietary allowance for adults (EITENMILLER 2004). However, the same serving of French fries can probably more often initiate *in vivo* and/or in the gastrointestinal tract losses of α -tocopherol originating from other dietary sources. These losses (i.e. the prooxidant capacity of the thermoxidised oil contained in the discussed serving of French fries reduced by the content of α -tocopherol in this serving) can range from 0 mg to 5 mg, i.e. up to 35% of the daily vitamin E recommended dietary allowance for adults. These estimates confirm a necessity of the increased intake of vitamin E in combination with consumption of fried foods to compensate a negative incidence of lipid oxidation products and to provide sufficiency of this vitamin (CHOW 2007). In this respect, increased intake of vitamin E has no negative effects *in vivo* (EITENMILLER 2004), in contrast to plant oils in which tocopherols exhibit the antioxidant activity only to a certain optimal concentration and when the tocopherol levels exceed this concentration, the antioxidant activity diminishes and the tocopherols exhibit a prooxidant behaviour (EVANS *et al.* 2002).

CONCLUSIONS

Though the obtained results represent preliminary calculations based on *in vitro* experiments (and must be verified under the conditions similar to conditions *in vivo* and/or in the gastrointestinal tract), they quite obviously illustrate significant health risks of fried foods in relation to the oxidative stress, as well as the protective action of sufficient or preferably increased intake of antioxidants (tocopherols above all). However, the presented results should be verified first of all using a more selective (probably HPLC) monitoring of tocopherol losses to eliminate the influence of mutual reactions between various antioxidants and other possible reactions affecting the antioxidant capacity (PAZOS *et al.* 2007).

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