Browning Reactions between Oxidised Vegetable Oils and Amino Acids

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Abstract

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Browning reactions of oxidised lipids with amino acids were studied in mixtures of refined soybean or rapeseed oil with alanine, valine, lysine, serine, cystine, cysteine, methionine, proline, and tryptophan. Oils were deposited in thin layers on cellulose fibres impregnated with the individual amino acids. The reaction proceeded in the dark, in dry air, at 50°C and at free access of oxygen. The browning determined at 430 nm followed a nearly zeroth order reaction without any induction period. The browning was very weak in the absence of amino acids, and all amino acids increased the browning rate, especially cysteine, methionine, and even more proline and tryptophan. The reaction rates were nearly the same in mixtures with rapeseed and soybean oils. Small amounts of hydroperoxides did not appreciably affect the browning rate. In the presence of copper ions, which belong to the most active catalysts of oxidation, the reaction rate was substantially higher. On the contrary, in the presence of antioxidants, the reaction rate was reduced to a marked degree but no induction period was observed. The probable main reaction mechanism was the reaction of lipid hydroperoxides, free radicals produced by their decomposition and/or unsaturated aldehydes under the formation of unsaturated imines which further polymerised into brown macromolecular substances.

Keywords: amino acids; autoxidation; browning, nonenzymic; hydroperoxides; imines; rapeseed oil; soybean oil

Nonenzymic browning reactions resulting from the interactions of amino acids or proteins with reducing sugars are considered either as positive or negative processes, depending on the particular food. However, nonenzymic browning reactions resulting from the interactions of amino acids or proteins with oxidised lipids are generally considered only as a negative process because of the impaired sensory quality.

The first observations on the colour deterioration of stored fish white muscle due to protein-oxidised lipids reactions were published more than 45 years ago (Stansby 1957). The mechanism of the browning reaction was studied on emulsions of menhaden oil in aqueous protein solutions (Venolia *et al.* 1957). Three parallel processes were recorded: (1) oxidation of polyunsaturated fatty acids bound in fish oil; (2) polymerisation of oxidation products into

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macromolecular compounds; (3) formation of brown pigments by reactions between different active groups of oxidising lipids and amine groups of proteins. The last process resulted in most intensive discolouration. Similar dark brown compounds were produced by reactions of proteins isolated from boiled dry anchovy, dry mackerel, and salty trout with autoxidising methyl linoleate (Fujimoto *et al.* 1968; Fujimoto 1970). Most brown products were soluble in polar organic solvents, and only a small part remained soluble in water (Fujimoto *et al.* 1971a, b). It seems that lipid-protein interactions are more important as browning precursors in fish muscle than Maillard reaction products formed from amino acids and ribose; however, Maillard products inhibited lipid oxidation (Toyomizu 1970). In mixtures of polyunsaturated fatty acid esters with carp, cod, or mackerel homogenates, the browning reaction proceeded via the formation of light coloured intermediary products, chiefly Schiff bases, which further polymerised into dark brown polymeric pigments (Pokorný *et al.* 1974a).

Browning reactions proceeded similarly in model systems containing dienoic and trienoic fatty acid esters and proteins, both in the presence and in the absence of water, e.g. insoluble brown substances were observed in model experiments with egg albumin (El-Tarras *et al.* 1971; Pokorný *et al.* 1974a, b). The reactions were very rapid under roasting and frying conditions but the browning processes were not negligible even at 40 or 60°C, of course after several days (Pokorný *et al.* 1973b). The browning intensity was found proportional to the peroxide value of the original oil (El-Zeany 1976).

Not only amino acids and proteins but also amines or ammonia participate in the browning reactions (Black *et al.* 1978). Primary amine groups are the reactive sites in protein molecules as protein treated with formaldehyde, by which the primary amine groups were converted into aminodimethylol derivatives, did not form dark brown products (Pokorný *et al.* 1975). The effect of the chemical structure of amino acids on the course of browning was studied in mixtures of amino acids with methyl linoleate on a cellulose support at 50°C (Uematsu & Ishii 1983a). The most intensive browning at 430 nm was observed in mixtures with methionine, glycine, proline, and serine after 10 days of storage, and with lysine after 18 days. The browning was significantly slower in mixtures with other amino acids. No browning occurred in a mixture with *N*-acetylglycine. The same experiments were carried out with Celite-purified peanut oil (Uematsu & Ishii 1983b). The browning was very intensive in mixtures with lysine, cysteine, and proline, less intensive with methionine and serine. Contrary to the experiments with methyl linoleate, the browning reaction was much slower with methionine and glycine; no reaction was observed in the mixture with *N*-acetylglycine. In analogous experiments carried out with 2, 4-decadienal – an important oxidation product of linoleic acid – instead of linoleic acid or peanut oil (Ishii *et al.* 1992), the browning reaction was also very intensive, particularly with alanine and proline, but nearly no reaction occurred with *N*-acetylglycine.

As the influences of the individual amino acids differed in the dependence on the lipidic precursor, we studied the browning reactions in mixtures with refined rapeseed and soybean oils, which are the most important edible oils in Central Europe. The reaction conditions were identical to those reported above (Uematsu & Ishii 1983a, b), which facilitates the comparison.

MATERIALS AND METHODS

Materials. Amino acids (purity 98–99%) were produced by Fluka, Chemie GmbH, Buchs, CH (L-lysine monohydrochloride; L-proline; L-alanine; L-methionine; L-tryptophan; DL-serine) and by Lachema-Ner s. r. o., Brno, plant Neratovice, CZ (L-cysteine, L-cystine); copper acetate monohydrate by Lachema; tert. butylated hydroxyanisole (BHA) by Sigma Chemical Co., St. Louis, MO, USA; methanol p. a. and *n*-hexane p. a. by Penta, Prague, CZ, producing plant Chrudim. The sweetgrass extract was prepared by the extraction of dry leaves (*Hierochloë odorata* Wahlnb.) with acetone (Bandonienė *et al.* 2000) followed by the removal of the solvent under reduced pressure; it contained 87.9 mg/g extract of total phenolic substances, expressed as gallic acid equivalents (equal to 8.8%), determined after Folin and Ciocalteu in a modification (Singleton *et al.* 1999).

Filter paper Whatman No. 42, diameter 110 mm (Whatman International Ltd., Maidstone, England, UK) was used for the browning experiments, while filter paper No. 390 of the diameter 90 mm (Spezialpapier Filtrak GmbH, Bärenstein, D) was used for the filtration of extracts.

Rapeseed and soybean oils were plant-scale refined in Setuza a. s., Ústí n. L., CZ, and stored in a refrigerator. Their properties are given in

Table 1. Properties of vegetable oils used in the experiments

Characteristic	Rapeseed oil	Soybean oil
Acid value (mg/g)	0.08	0.07
Peroxide value (meq/kg)	0.36	1.29
Saturated fatty acids (%)	7	14
Monoenoic fatty acids (%)	61	23
Dienoic fatty acids (%)	21	56
Trienoic fatty acids (%)	11	7
Tocopherols (mg/kg)	1150	1050

Table 1. Oxidised oils were prepared by keeping the above oils in a thermostated oven in the dark at 60°C for 98 h (soybean oil, the final peroxide value 14.1 meq/kg) or 190 h (rapeseed oil, the final peroxide value 24.8 meq/kg), respectively.

Analytical methods. The absorbance of methanol extracts was measured at 430 nm in 40.0 mm silica cells (Spektromom, Budapest, H). The peroxide value was determined iodometrically using the standard procedure (AOCS 1998). The linear and logarithmic regressions, correlation coefficients, and standard deviations were calculated using the MS Statistica 5.0 software. The probability level was $P = 0.95$.

Procedure. A piece of filter paper Whatman No. 42 (diameter 110 mm) was impregnated with 25 or 50 ml of aqueous solution containing 1 mmol amino acid (heated up to 80°C, if necessary), and dried in a current of warm air. The dry paper was then impregnated with a solution of 300 mg soybean or rapeseed oil dissolved in 5 ml hexane, and dried in a stream of warm air. A series of 10 paper specimens was prepared at the same time. Each paper sample was stored in a thermostated oven at 50°C in the dark under free access of air. At defined time intervals, the sample was taken out of the oven, cut into small pieces, and shaken with 10 ml methanol for 15 min. The extract was filtered through filter paper No. 390 (diameter 90 mm) into a 25.0 ml measuring flask. The procedure was repeated once more with another 10 ml methanol, and for the third time with 8 ml methanol. The flask and the filter were then washed with 3 ml methanol. The measuring flask was then filled up with methanol, and the absorbance of the final solution was measured at 430 nm in 40 mm quartz cells.

RESULTS

The optimum reaction conditions were checked in preliminary experiments under the conditions used by Uematsu and Ishii (1983a). Mixtures of refined edible oil with 1 mmol lysine were tested. From the standpoint of reaction rates, the temperature of 50°C was confirmed as more suitable than those of 40 and 60°C. The impregnation of Whatman No. 42 filter paper (diameter 110 mm) with 300 mg of oil dissolved in hexane was appropriate to obtain a homogenous film of oil, as compared to 400 mg. The same amount of oil was used in the earlier experiments (Uematsu & Ishii 1983b). Methanol as a solvent for the extraction of yellow and brown pigments was found more efficient than petroleum ether, diethyl ether, hexane, or ethyl acetate. Different volumes of the solvent for the extraction were tested in the range of 10–100 ml; 25.0 ml was found optimal. The absorbance measured at 430 nm was low but still acceptable if measured in 40 mm cells. The absorbance was approximately twice as high in the low maximum at 360 nm, but the position was already outside the visible spectrum. Another, much higher maximum was observed at 278 nm, however, other components, such as tocopherols or other antioxidants interfered here. The standard deviation of parallel experiments was 0.008 after 2.9 days at 50°C and 0.005 after 6.68 days. The experiments published by Uematsu and Ishii (1983a) using mixtures of methyl linoleate with cysteine, proline, or lysine were repeated and found to resemble those given in the above paper.

Results of the experiments with rapeseed and soybean oils are summarised in Tables 2 and 3, respectively. The main experiments were carried out with oils of low peroxide value, corresponding to those of oils suitable for consumption, i.e. in the range of 0.41–1.53 meq/kg in the case of rapeseed oil, and in the range of 2.70–3.11 meq/kg in the case of soybean oil. As the rate might be affected by the hydroperoxide content (El-Zeany 1976), some experiments were repeated with oils containing higher concentrations of hydroperoxides (24.8 meq/kg in rapeseed oil and 14.1 meq/kg in soybean oil). The degree of browning was determined after 10 different reaction times, using different filter paper samples each time. Each run was repeated at least twice in the case of greater differences up to five times.

The time dependence of the transmittances or absorbances at 430 nm showed to be almost a linear

Table 2. Browning reactions in mixtures of rapeseed oil with amino acids

Amino acid	Initial peroxide value	Correlation coefficient[a] r	Mean slope[b] (×1000)	Standard deviation[c] (×1000)
Blank	1.53	0.97	0.9	0.1
Alanine	0.41	0.97	2.0	0.4
Valine	0.49	0.96	3.9	0.2
Lysine	0.41	0.96	1.4	0.3
Serine	0.49	0.86	2.1	0.4
Cysteine	0.41	0.96	4.4	0.5
Cystine	0.49	0.92	2.2	0.7
Methionine	1.53	1.00	4.7	0.5
Proline	0.50	0.99	20.0	1.0
Tryptophan	0.49	0.97	20.0	0.6
Alanine	24.8	0.98	2.4	0.2
Lysine	24.8	0.98	1.2	0.2
Cysteine	24.8	0.90	4.4	0.3
Proline	24.8	0.98	10.0	0.8

[a]correlation coefficient of linear regression between time and absorbance; [b]slope of the linear time dependence of absorbance; [c]standard deviation of the slope constant

Table 3. Browning reactions in mixtures of soybean oil with amino acids

Amino acid	Initial peroxide value	Correlation coefficient[a] r	Mean slope[b] (×1000)	Standard deviation[c] (×1000)
Blank	3.11	0.94	1.1	0.1
Alanine	2.70	0.97	4.0	0.3
Valine	3.11	0.99	4.0	0.3
Lysine	2.70	0.98	3.0	0.1
Serine	3.11	0.92	1.2	0.2
Cysteine	2.70	0.97	3.9	0.4
Cystine	3.11	0.98	1.8	0.2
Methionine	3.11	0.99	4.0	0.5
Proline	2.90	0.98	20.2	1.0
Tryptophan	3.11	0.98	30.1	0.2
Alanine	14.1	0.93	2.4	0.1
Lysine	14.1	0.94	1.4	0.2
Cysteine	14.1	0.96	7.2	0.2
Proline	14.1	0.93	8.0	0.4

[a,b,c] see Table 2

function. Deviations from the linearity were negligible in the time range studied. Correlation coefficients of the absorbance/time or transmittance/time slopes were very similar, in the range of r = –1.00 and –0.86 in the case of transmittances, and in the range of r = 1.00 and 0.86 in the case of absorban-

Table 4. Effect of antioxidants on the browning rate

Vegetable oil	Antioxidant	Amino acid	Correlation coefficient[a] r	Mean slope[b] (×1000)	Standard deviation[c] (×1000)
Rapeseed	BHA	Cysteine	0.94	2.0	0.6
	BHA	Proline	0.99	13.7	1.4
	SG	Cysteine	0.93	1.7	0.3
	SG	Proline	0.99	11.4	1.1
Soybean	BHA	Cysteine	0.98	1.2	0.3
	SG	Proline	0.96	1.2	0.4

[a,b,c] see Table 2; BHA = 0.02% butylated hydroxyanisole; SG = 0.1% sweetgrass extract

ces in rapeseed oil (the average values of r = –0.96 and r = 0.97, respectively). In the experiments with soybean oil, the range of r = –0.99 and –9.90 was obtained in the case of transmittances (the average value of r = –0.95) and the range of r = 0.99 and 0.93 in the case of absorbances (the average value of r = 0.97). As the differences were not significant, only the correlation coefficients of absorbance/time dependence are given in Tables 2 and 3.

In some experiments, semilogarithmic regressions were obtained as slightly better fitting the experimental data but the differences in R^2 values were not significant (P = 0.95). Therefore, only the data for linear regressions have been included in Tables 2 and 3.

The slopes (b) of the time/absorbance plots: Absorbance = $a + b.t$, where t = time (h), are proportional to the browning rate (Tables 2 and 3). The values obtained in the analysis of mixtures with rapeseed oil were not significantly different from those of mixtures with soybean oil, with the exception of mixtures with tryptophan. Values obtained with moderately oxidised oils were not statistically different from those obtained with fresh oils.

The browning rates in mixtures containing antioxidants were substantially lower than those in mixtures containing natural tocopherols only (Table 4), both in the case of synthetic tert. butylated hydroxyanisole and on that of sweetgrass extract as a representative of natural antioxidants. On the contrary, the reaction rates were substantially higher in mixtures containing copper ions as a representative of metallic prooxidants. The increase did not depend on the type of the oil present, and it was pronounced in the case of both lysine and proline.

DISCUSSION

The course of browning followed nearly a zero order kinetics (Tables 2 and 3). Such kinetics could be expected as the browning reactions are very complex and the concentration of precursors (i. e. amino acids and oxidised polyenoic fatty acids) did not change appreciably during the early reaction stage studied in our experiments. In most studies published in the literature (referred in Pokorný *et al.* 1973a, 1975; Pokorný & Kołakowska 2002) proteins were mixed with polyunsaturated fatty acid esters so that only the free amine groups of proteins could react with oxidised lipids.

The concentration of amine groups is much higher in mixtures with free amino acids. The α-amino acids can be also decarboxylated into amines by hydrogen peroxide (Nilov & Ogorodnik 1967), alkyl hydroperoxide (Vilyanskaya *et al.* 1969), or lipid hydroperoxides (Uematsu *et al.* 1986) by a mechanism analogous to the Strecker degradation. Therefore, the browning reactions were rather rapid in mixtures of amino acids with methyl linoleate (Uematsu & Ishii 1983a) or in mixtures with tocopherol-free peanut oil (Uematsu & Ishii 1983b). However, vegetable oils usually contain natural antioxidants, particularly tocopherols. Therefore, we studied mixtures of amino acids with refined rapeseed oil or soybean oil which are the most common edible oils in Western and Central Europe. Both oils contained tocopherols in the amounts common in refined oils. Both oils were relatively rich in tocopherols, mainly γ-tocopherol and α-tocopherol (Table 1). Cellulose was used as a support, similarly as in the above papers by Uematsu and Ishii (1983a, b), because its effect on

the browning reactions is nearly neutral (Pokorný 1981; Pokorný & Kołakowska 2002).

The intensity of the browning reactions increases with the increasing degree of unsaturation (Pokorný *et al.* 1973a, 1976) as was shown with methyl linoleate and methyl linolenate under the same conditions. Surprisingly, nearly the same rates of browning were obtained in the case of rapeseed oil as in the case of soybean oil in our experiments (Tables 2 and 3) in spite of the substantially higher content of linoleic acid in soybean oil as compared to rapeseed oil. Perhaps, the higher content of linoleic acid in soybean oil was compensated by the moderately higher content of linolenic acid in rapeseed oil as found in our previous experiments on oil/protein mixtures (Uematsu *et al.* 2002).

Lipids spread in a thin layer on the surface of cellulose fibres are exposed to the free access of air oxygen so that the oxidation reactions are relatively rapid. Polyunsturated fatty acids are oxidised into hydroperoxides which react with amino acids forming unsaturated imines (Figure 1). Similar reactions may proceed with free radicals formed by hydroperoxide decomposition. The reaction is accompanied by decarboxylation of the respective amino acid, i. e. it is analogous to Strecker degradation (Uematsu *et al.* 1986; Pokorný 1981; Pokorný & Kołakowska 2002). Oxidised fatty acid hydroperoxides are further cleaved into unsaturated aldehydes which, again, are active precursors of the browning reactions after condensation with amine groups (Ishii *et al.* 1992). Unsaturated yellow coloured imines formed in all these reactions (Figure 1) are slowly transformed into brown macromolecular substances by subsequent polymerisation and polycondensation reactions (Pokorný & Sakurai 2002).

In mixtures with methyl linoleate free of antioxidants, the autoxidation was very rapid so that deep browning was observed as early as after 10 days

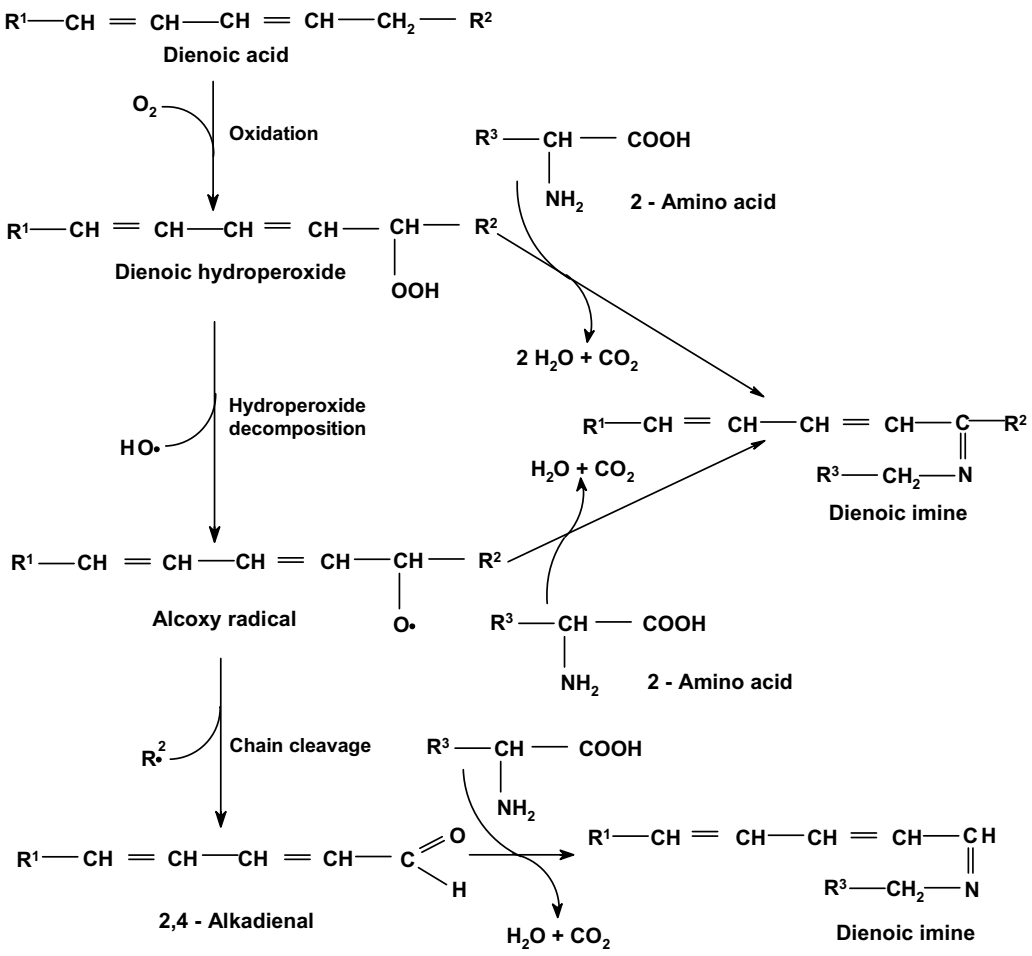

Figure 1. Reaction mechanism of oxidised lipids with amino acids

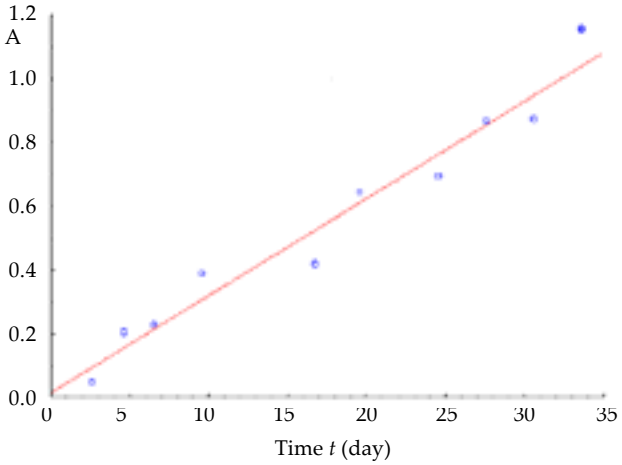

Figure 2. Reaction course of browning in a mixture of soybean oil with tryptophan

A = absorbance at 430 nm; t = reaction time at 50°C (days); the linear expression: A = 0.020 + 0.030t; N = 10; r = 0.98; soybean oil of initial peroxide value of 2.83 meq/kg

(Uematsu & Ishii 1983a). Some amino acids inhibited the oxidation while cysteine, phenylalanine, and lysine promoted it. The same amino acids inhibited the browning, as well. In mixtures of glycine with Celite- or Florisil-treated peanut oil, both the hydroperoxide content and the browning intensity rose slowly for 24 days but it started to increase rapidly afterwards (Uematsu & Ishii 1983b). In our experiments with refined vegetable oils containing tocopherols as antioxidants, the browning reaction was slower than in Florisil-treated oil, and the end of the induction period was not attained, at least not during the time period studied. An example of the course of browning is shown in Figure 2. Similar time/absorbance courses were observed in other experiments.

The linear course of browning is probably in connection with the nearly constant initiation rate, due to the presence of tocopherols. The concentration of oxygen was also constant as the amount of oxygen which reacted with free radicals was rapidly supplied in the oil layer by diffusion from air. Another factors were the complexity of reactions between the oxidation products and amino acids, and only moderate changes of reactants. The interphase between the lipid phase and amino acids, where the reactions proceeded, was also nearly the same. A linear course of oxygen absorption and the weight increase during the induction period was observed in our other experiments under similar conditions (unpublished).

As explained above, the browning obviously results from the reactions of oxidation products with amino acids, therefore, the influence of the hydroperoxide content on the browning rate can be expected. Minor differences in peroxide values of the oil phase did not, however, substantially influence the reaction rate (Tables 2 and 3, compare the upper and lower results) due to the natural content of tocopherols. If additional antioxidants were added to oils containing tocopherols, the browning rate was substantially suppressed (Table 4), yet the browning started from the beginning of reaction and no induction period was observed. The probable explanation is that the combination of antioxidants only reduced the formation of hydroperoxides and/or their decomposition; the reaction course studied was thus again proceeding only in a part of the induction period. A similar effect of antioxidants on the browning inhibition was

Table 5. Effect of prooxidants on the browning rate

Vegetable oil	Amino acid	Correlation coefficient[a] r	Mean slope[b] (× 1000)	Standard deviation[c] (× 1000)
Rapeseed oil[d]	alanine	0.96	29.0	1.5
	proline	0.96	50.6	0.8
	cysteine	0.99	30.2	0.8
Soybean oil[e]	alanine	0.99	20.2	1.4
	proline	0.96	37.2	0.3

[a,b,c]see Table 2; [d]initial peroxide value 0.50 meq/kg; [e]initial peroxide value 3.11 meq/kg; 1 mg copper acetate added to each experiment

also observed in lipid-protein mixtures (El-Zeany *et al.* 1975). Several natural antioxidants from spices inhibit the oxidation of polyunsaturated vegetable oils (Pokorný *et al.* 2001), and the same could be expected in the case of browning reactions. The addition of a synthetic antioxidant produced the same effect as the addition of a natural antioxidant, i. e. sweetgrass extract, which was found very active in edible oils (Zainuddin *et al.* 2002).

On the contrary, ions of transient valency metals, such as copper, increase the rate of hydroperoxide decomposition, thus enhancing the formation of free radicals, and, consequently, oxidation. In our experiments, the presence of copper ions substantially increased the browning rate in the presence of amino acids (Table 5). This effect is in agreement with the expectations and with our earlier experiments with mixtures of fish oil methyl esters and proteins (El-Zeany *et al.* 1974).

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Souhrn

Hutapea E. B., Parkányiová L., Parkányiová J., Miyahara M., Sakurai H., Pokorný J. (2004): **Reakce hnědnutí mezi oxidovanými rostlinnými oleji a aminokyselinami.** Czech J. Food Sci., **22**: 99–107.

Reakce hnědnutí byly zkoumány ve směsích aminokyselin alaninu, valinu, lysinu, serinu, cystinu, cysteinu, methioninu, prolinu a tryptofanu s rafinovaným řepkovým a sójovým olejem. Oleje byly naneseny v tenké vrstvě na celulosová vlákna impregnovaná aminokyselinami. Reakce probíhala v suchém prostředí, za tmy při 50 °C a za volného přístupu vzduchu. Intenzita zbarvení při 430 nm rostla lineárně v závislosti na době záhřevu. Nebyla pozorována žádná indukční perioda. Hnědnutí v samotném oleji bez aminokyselin bylo zanedbatelné, ale všechny aminokyseliny urychlovaly hnědnutí, zvláště cystein a methionin a ještě více prolin a tryptofan. Malé množství hydroperoxidů nemělo vliv na reakční rychlost, ale měďnaté ionty, které patří k nejúčinnějším katalyzátorům oxidace, také výrazně urychlovaly hnědnutí. Naproti tomu antioxidanty hnědnutí zpomalovaly, aniž působily vznik indukční periody. Z výsledků plyne, že je potvrzen mechanismus tvorby nenasycených iminů jako meziproduktů aminokyselin nebo produktů Streckerovy degradace s hydroperoxidy nebo volnými radikály a nenasycenými aldehydy vznikajícími při jejich rozkladu. Iminy dále polymerují na hnědé makromolekulární sloučeniny.

Klíčová slova: aminokyseliny; autooxidace; hnědnutí neenzymové; hydroperoxidy; iminy; řepkový olej; sójový olej

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