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Antioxidant Activity of N-(2-Pyron-3-yl)-α-L-aspartyl-L-phenylalanine Methyl Ester*

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Abstract

KOGA H., TOKUYAMA T., SAKURAI H., RÉBLOVÁ Z., TROJÁKOVÁ L., NGUYEN H. T. T., POKORNÝ J. (1999): **Antioxidant activity of N-(2-pyron-3-yl)-α-L-aspartyl-L-phenylalanine methyl ester**. Czech J. Food Sci., 17: 41–48.

During the storage or heating of food products and beverages containing ascorbic acid and Aspartame, Maillard reactions occur, and N-(2-pyron-3-yl)-α-L-aspartyl-L-phenylalanine methyl ester (NPA) is the major product. The compound shows the antioxidant activity, which was more evident in reducing the rate of hydroperoxide accumulation than in prolonging the induction period. It was higher than that of α-tocopherol in crude peanut oil at 60 °C, and the activity was still pronounced at 97 °C, however, the efficiency was very low in refined rapeseed oil at 40 °C. The efficiency decreased with increasing reaction temperature. The activity became very low at frying temperatures both in peanut oil and in rapeseed oil. Moderate synergistic effect was observed between pyrone and both *tert.* butylhydroxytoluene and *tert.* butylhydroxyanisole under storage conditions. The presence of pyrone slightly increased the stability of rapeseed oil in mixture with starch and egg albumin, but the antioxidative effect was lost in the presence of water. The decomposition of pyrone during heating could contribute to the discolouration of the oil fraction.

Key words: antioxidant activity; ascorbic acid; aspartame; browning; discolouration; hydroperoxides; N-(2-pyron-3-yl)-aspartylphenylalanine methyl ester; peanut oil; rapeseed oil

Kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone) is a natural food component as it is produced by many species of microorganisms, common in food industry, such as *Aspergillus* and *Penicillium*. Kojic acid was found (WEI *et al.* 1991) to act as an *in vivo* antioxidant in inhibition of polyphenol oxidases in food products. It shows antioxidant activity in fats and oils as well, particularly after conversion into alkyl and O-acyl derivatives, which are more liposoluble (ABE, TAKASHI 1970); *tert.* butylhydroxyanisole (BHA) and citric acid act as synergists. The structurally related 3-hydroxy-2-pyrone (HPY) was found efficient as an antioxidant in peanut and soybean oils and in lard (TAKIZAWA *et al.* 1984). In vegetable oils, tocopherols showed synergistic activity, but HPY was found active even in oils stabilized after preliminary removal of tocopherols. If HPY was present at higher concentrations, its activity was comparable with that of BHA.

The structurally related N-(pyron-3-yl)-α-L-aspartyl-L-phenylalanine methyl ester (NPA) is formed by interaction of Aspartame with dehydroascorbic acid, formed from L-ascorbic acid during heating or storage of foods and beverages (SAKURAI *et al.* 1996). Therefore its antioxidant activity was tested in fats, oils and in simulated food products.

MATERIAL AND METHODS

Material

Aspartame (L-aspartyl-L-phenylalanine methyl ester) was produced by Pure Wako Chemical Industries, Japan; L-dehydroascorbic acid was prepared by oxidation of ascorbic acid with silver oxide, and a subsequent purification (ISHII *et al.* 1968; SAKURAI *et al.* 1985). It was reacted with Aspartame by heating to 90 °C; reaction products were fractionated by crystallization.

Crude peanut oil was prepared by crushing peanuts, and extracting thrice with hexane at ambient temperature, each time for 4 hours. The miscella was heated in a rotary

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evaporator at 40 °C to remove hexane; refined zero-erucic rapeseed oil was produced from a mixture of expeller pressed and solvent extracted crude oils by commercial superdegumming and alkali refining. Wheat starch (manufactured by Starch Research Institute, Havlíčkův Brod, Czech Republic) and egg albumin (manufactured by Lachema, Brno, Czech Republic) were used in air-dry conditions. The synthetic antioxidants – *tert.* butylhydroxytoluene (BHT) and *tert.* butylhydroxyanisole (BHA) – were products of Nacalai Tesque Inc., Japan, and α-tocopherol was produced by Eisai & Co., Japan.

Methods

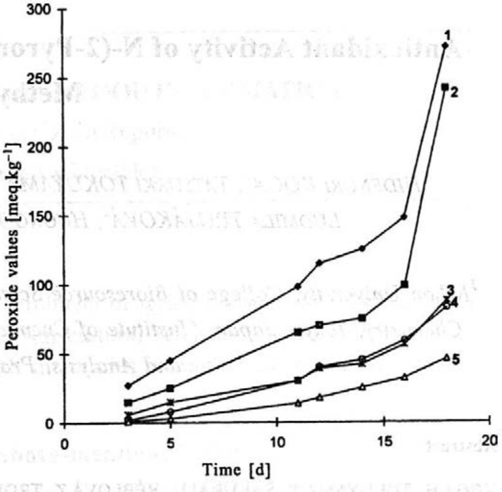
The peroxide value was determined iodometrically according to the international standard procedure (AOCS 1996); in agreement with the standard, the peroxide value is expressed in mval/kg, where 1 mval = 0.5 mmol. The carbonyl value was determined spectrophotometrically using the 2,4-dinitrophenylhydrazone procedure, measured as the quinoid (JOCS 1996 – method 2.54). The acid value was determined by titration (AOCS 1996), and is expressed in mg KOH per g of fat. The ultraviolet spectra were measured in hexane solution, using the apparatus PU 8740 UV/VIS, manufactured by Philips, UK. Tocopherols were determined by capillary gas chromatography following the standard method (IUPAC 1987).

The following accelerated methods were used for the determination of resistance to oxidation: A = Schaal oven test at 60 °C (the peroxide value as an indicator) or 40 °C with gravimetric detection (POKORNÝ *et al.* 1985); B = Active Oxygen Method at 97 °C (JOCS 1996 – Method 2.5.1), a boiling water bath was used, and the flow rate of air was 2.33 ml/s; the tube diameter was 18 mm, and the oil content 15 g; at 115 °C and 130 °C (AOCS 1996) a silicone bath was used; C = heating in test tubes in an oil bath at 180 °C, determining the acid, peroxide and carbonyl values; D = heating in the Oxipres ML apparatus (Mikrolab Aarhus, Denmark) at 100 °C, unless otherwise stated, 5g sample, pressure 0.5 MPa, without stirring, and measuring the decrease of oxygen pressure. The end of the induction period is determined as the time, after which the weight or other indicator of oxidation increases over the initial value by the value significantly different from zero. The rate of peroxide increase was calculated using expressions for a first order kinetics.

RESULTS AND DISCUSSION

Experiments using the Procedure A

The antioxidant efficiency of NPA was compared with other antioxidants (BHA and BHT) in crude peanut oil (Fig. 1) at 60 °C. NPA was found less active than BHT, but still significantly superior to α-tocopherol. It should be acknowledged that some natural tocopherols were present in crude peanut oil (55 mg α-tocopherol, 1 mg β-tocopherol, 67 mg γ-tocopherol and 2 mg δ-tocopherol in 1 kg of oil) and thus the activities of the antioxidants tested were affected by their synergism. In this experiment, the antioxidant activity was more pronounced in reducing the rate of peroxide increase during the induction period (which is important for the start of flavour deterioration) than in prolonging the induction period (Table 1). The sample stabilized with 0.02 % NPA showed no significant improvement of oxidative stability, but the addition of 0.04 % NPA substantially lowered the peroxide accumulation during the induction period, and even prolonged the induction period to a moderate degree. The addition of α-tocopherol acted prooxidatively, which may be attributed to the content higher than optimal. It was observed (HEIMANN, PEZOLD 1959; KAMAL-ELDIN & APPELQVIST 1996) that edible oils containing higher amounts of tocopherols had lower oxidative stability than oils with their natural, lower tocopherol content. The most active antioxidant was BHT.



1 – without antioxidants; 2 – 0.03% α-tocopherol; 3 – 0.02% BHT; 4 – 0.02% NPA; 5 – 0.04% NPA

Fig. 1. Antioxidant effect of NPA in crude peanut oil at 60 °C

The antioxidant activity of NPA was also tested in rapeseed oil (Fig. 2); in these experiments, the temperature

Table 1. Antioxidant activity of NPA in crude peanut oil

Antioxidant added	Induction period [d]	Rate of peroxide increase [mval.g^{-1}.d^{-1}]
Control	14.7	5.0
0.03% α-tocopherol	15.0	8.5
0.02% BHT	>20.0	8.6
0.02% NPA	14.8	4.0
0.04% NPA	15.7	2.8

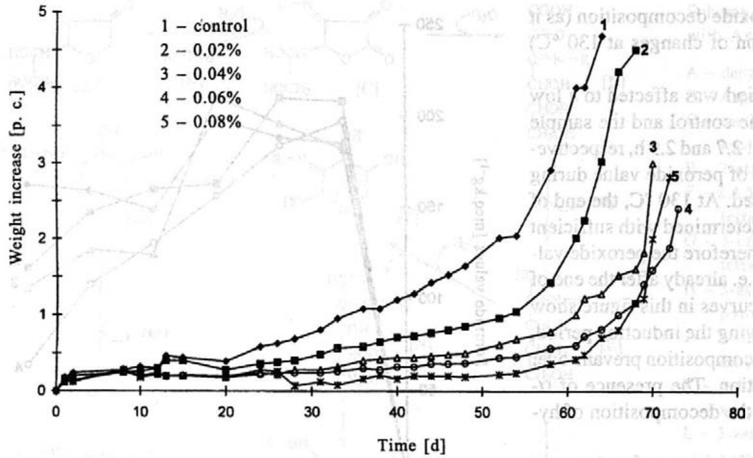


Fig. 2. Effect of NPA on the oxidation of refined rapeseed oil at 40 °C

was kept at 40 °C as linolenic acid hydroperoxides, which are formed during the induction period, are unstable at 60 °C. Under these conditions, NPA inhibited the absorption of oxygen and the weight increase during the induction period (Fig. 2 and Table 2), but the effect on the induction period was only moderate again.

Table 2. Effect of the concentration of NPA on the stability of rapeseed oil against oxidation at 40 °C

Concentration of NPA [p. c.]	Induction period [d]	Rate of oxidation [p. c. of weight increase.d^{-1}]
0	56.7	2.4
0.02	61.3	1.3
0.04	68.4	0.7
0.08	68.2	0.2

The efficiency depended on the concentration of NPA, which can be explained by low solubility of NPA in oils. The antioxidant activity is effected mostly on the interphase between the undissolved NPA and the oil phase.

Experiments using the Procedure B

The use of the Active Oxygen Method differs from the Schaal Oven Test in a better contact of oil with oxygen and in continuous stirring of the sample with a stream of air. Oil gets in a better contact with undissolved fraction of the antioxidant. The results obtained at 97 °C are given in Fig. 3. Under these conditions, the antioxidant activity did not consist in the prolongation of induction period, but in reduction of the formation or accumulation of hydroperoxides during the induction period, which is very important for flavour stability. The rates of peroxide changes are shown in Table 3. They are probably more influ-

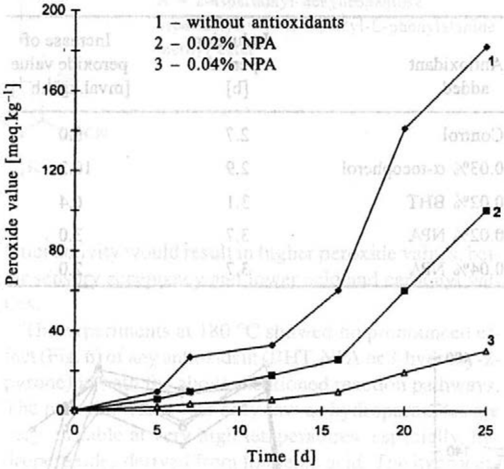


Fig. 3. Effect of NPA on the oxidation of crude peanut oil at 97 °C

Table 3. Effect of NPA on the resistance of crude peanut oil against oxidation under the conditions of the AOM procedure at 97 °C

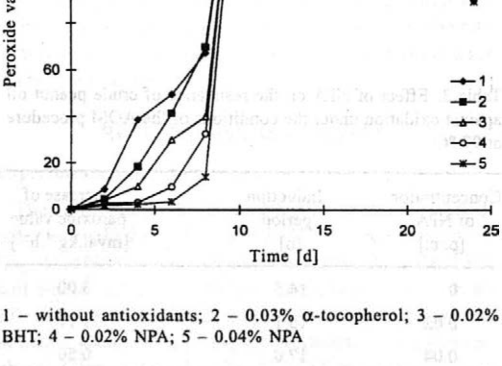
Concentration of NPA [p. c.]	Induction period [h]	Increase of peroxide value [mval.kg^{-1}.h^{-1}]
0	14.5	3.00
0.02	15.1	1.17
0.04	17.0	0.50

enced by more rapid hydroperoxide decomposition (as it is shown later, at the discussion of changes at 130 °C) than by their slower formation.

At 115 °C, the induction period was affected to a low degree only by antioxidants, the control and the sample containing 0.03 % α-tocopherol: 2.7 and 2.9 h, respectively (Fig. 4 and Table 4); the rise of peroxide value during the induction period was reduced. At 130 °C, the end of induction period could not be determined with sufficient accuracy as it was very short, therefore the peroxide value was recorded only after 4 h, i.e. already after the end of induction period (Fig. 5). The curves in this figure show only changes at the stage following the induction period, when the process of peroxide decomposition prevails over the process of peroxide formation. The presence of α-tocopherol and NPA enhanced the decomposition of hydroperoxides in oxidizing oil (0.9 mval/kg.h in the control experiment and 6.0 mval/kg.h in the presence of 0.03% of α-tocopherol). In the presence of 0.02% and 0.04% NPA, the rate of peroxide decrease was 4.5 and 4.8 mval/kg.h, respectively. The hydroperoxide-decomposing activity may be due to the presence of nitrogen groups (TOTANI 1980; IIO *et al.* 1988; POKORNÝ 1987) in NPA or its decomposition product – aspartyl-phenylalanyl anhydride (Reaction scheme 1). The decomposition of NPA after Scheme 1 could contribute to the discolouration of peanut oil by formation of substances absorbing between 360–430 nm.

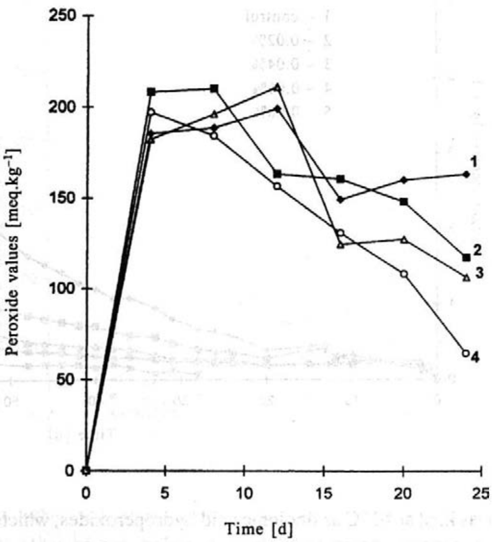
Table 4. Effect of antioxidants on the resistance of crude peanut oil against oxidation under the conditions of the AOM method at 115 °C

Antioxidant added	Induction period [h]	Increase of peroxide value [mval.kg^{-1}.h^{-1}]
Control	2.7	9.0
0.03% α-tocopherol	2.9	10.3
0.02% BHT	3.1	6.4
0.02% NPA	3.7	3.0
0.04% NPA	3.7	2.0



1 – without antioxidants; 2 – 0.03% α-tocopherol; 3 – 0.02% BHT; 4 – 0.02% NPA; 5 – 0.04% NPA

Fig. 4. Antioxidant effect of NPA in crude peanut oil at 115 °C

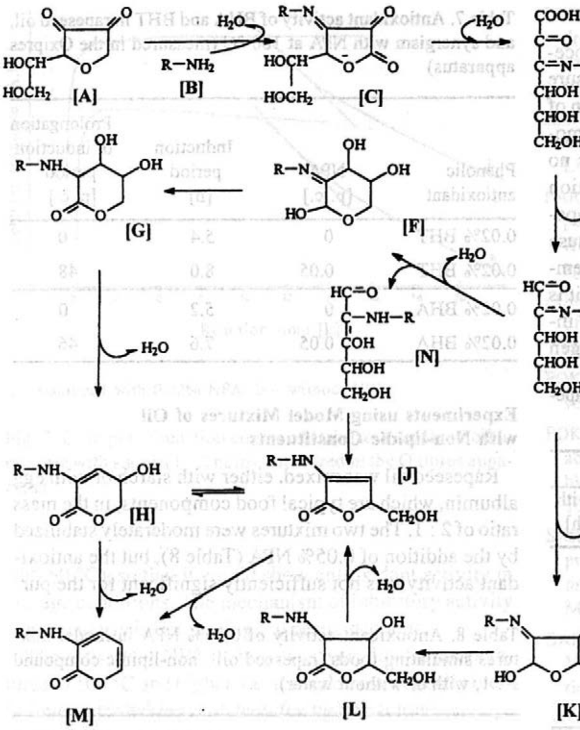


1 – without antioxidants; 2 – 0.03% α-tocopherol; 3 – 0.02% NPA; 4 – 0.04% NPA

Fig. 5. Antioxidant effect of NPA in crude peanut oil at 130 °C in the stage of oxidation following the maximum peroxide content

Experiments using the Procedure C

In these experiments, the availability of oxygen became the main factor influencing the reaction rate (because of nearly immediate consumption of oxygen, entering oil by diffusion from atmosphere). As the access of oxygen by diffusion from the atmosphere could be expected as constant, no antioxidant activity was expected in the classical way. The antioxidant activity is due to the activity of an antioxidant to react with free peroxy or alkoxy radicals stopping thus the reaction chain. As oxygen is missing in the system, the reaction chain would stop anyway. There is another possibility of antioxidant activity: Antioxidants may stabilize hydroperoxides already formed, and thus inhibit the formation of secondary reaction products which cause sensory rancidity (FRANKEL *et al.* 1989, 1994). The



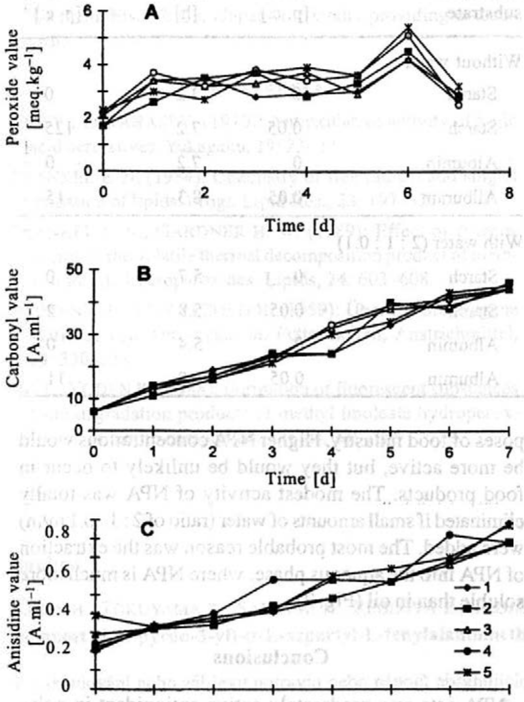
Schema 1. Reaction scheme of ascorbic acid with Aspartame

A – dehydroascorbic acid
B – Aspartame
C – condensation product with dehydroascorbic acid
E – 2-aspartamyliminopentose
F – 2,4,5-trihydroxy-3-iminoaspartamyl-tetrahydropyrane
G – 3-iminoaspartamyl-4,5-dihydroxy-5-tetrahydropyrone
H – 3-aspartamyl-4-hydroxy-dihydro-2-pyrone
J – 3-aspartamyl-5-(hydroxymethyl)-dihydrofuran-2-one
K – 2,4-dihydroxy-3-aspartamyl-5-(hydroxymethyl)-tetrahydrofuran
L – 3-aspartamyl-3-hydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-one
M – 3-aspartamyl-2-pyrone

A = 2-aspartamyl-dehydropentose
aspartamyl = A-α-aspartyl-L-phenylalanine methyl ester

latter activity would result in higher peroxide values, better sensory acceptancy and lower acid and carbonyl values.

The experiments at 180 °C showed no pronounced effect (Fig. 6) of any antioxidant (BHT, NPA or 3-hydroxy-2-pyrone) in both the above mentioned reaction pathways. The peroxide value was only low as hydroperoxides are very unstable at very high temperatures, especially, hydroperoxides derived from linolenic acid. The hydroperoxide stabilizing activity of NPA or of hydroxypyrone was not confirmed, at least at very high temperatures; however, oils oxidized at 180 °C do not contain only hydroperoxides (PAŘÍZKOVÁ *et al.* 1985; POKORNÝ *et al.* 1985). Various other peroxidic substances, such as cyclic or dimeric peroxides, prevail in such oils, which are more stable at 180 °C than hydroperoxides, but still can react with iodide. The effect of antioxidants on hydroperoxides may thus be masked by the interfering non-hydroperoxidic peroxides. Secondary reaction products could not be much affected by the presence of antioxidants either.



A – changes of the peroxide value; B – changes of the carbonyl value; C – changes of the *p*-anisidine value; 1 – without antioxidants; 2 – 0.02% BHT; 3 – 0.02% NPA; 4 – 0.04% NPA; 5 – 0.02% HPY

Fig. 6. Effect of NPA on the oxidation stability of refined rapeseed oil at 180 °C

Experiments using the Procedure D

The procedure differs from the three previous procedures as the reaction proceeds at higher oxygen pressure (0.5 MPa in our experiments) and that the consumption of oxygen (not the peroxide value) is recorded. The thermolability of hydroperoxides at high temperatures has no effect on the recorded results, expressed as induction periods. These experiments were carried out with rapeseed oil, which is particularly difficult to stabilize because of relatively high linolenic acid content. The effect of temperature on the induction period is shown in Table 5; it is evident that the antioxidant activity decreased with increasing reaction temperature. The diffusion of oxygen into oil from the gaseous phase is the rate-determining factor in the Procedure D as well as in the previous method (Procedure C). Therefore the prolongation of the induction period would become only moderate by addition of any antioxidant, not only NPA. The same is valid for the effect of concentration of NPA (Table 6), which could be considered as an additional proof of the relative inactivity of NPA under these extreme conditions.

Table 5. Effect of temperature on the induction period in rapeseed oil, measured in the Oxipres apparatus

Temperature [°C]	Induction period of rapeseed oil [h]	oil stabilized with 0.05% NPA [h]
80	36.0	40.4
100	3.6	5.1
110	5.2	8.0
120	1.8	2.0
150	0.55	0.55

Table 6. Effect of NPA concentration on its antioxidant activity in rapeseed oil at 100 °C (measured in the Oxipres apparatus)

Concentration of NPA [p. c.]	Induction period [h]	Prolongation of the induction period [p. c.]
0	3.6	0
0.02	3.4	0
0.05	6.4	77
0.10	5.3	47

Nevertheless, BHA and BHT had more pronounced antioxidant activities in rapeseed oil under the same conditions (Table 7); NPA had no practically important synergistic activity in their presence.

Table 7. Antioxidant activity of BHA and BHT in rapeseed oil, and synergism with NPA at 100 °C (measured in the Oxipres apparatus)

Phenolic antioxidant	NPA [p. c.]	Induction period [h]	Prolongation of induction period [p. c.]
0.02% BHT	0	5.4	0
0.02% BHT	0.05	8.0	48
0.02% BHA	0	5.2	0
0.02% BHA	0.05	7.6	46

Experiments using Model Mixtures of Oil with Non-lipidic Constituents

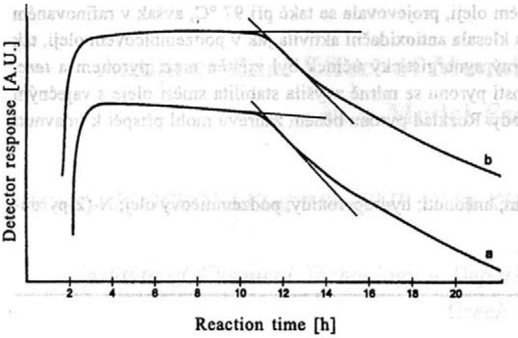
Rapeseed oil was mixed, either with starch or with egg albumin, which are typical food components, in the mass ratio of 2 : 1. The two mixtures were moderately stabilized by the addition of 0.05% NPA (Table 8), but the antioxidant activity was not sufficiently significant for the purposes of food industry. Higher NPA concentrations would be more active, but they would be unlikely to occur in food products. The modest activity of NPA was totally eliminated if small amounts of water (ratio of 2 : 1 : 0.1 m/m) were added. The most probable reason was the extraction of NPA into the aqueous phase, where NPA is much more soluble than in oil (Fig. 7).

Table 8. Antioxidant activity of 0.05% NPA in model mixtures simulating foods (rapeseed oil : non-lipidic compound 2 : 1, with or without water)

Non-lipidic substrate	NPA [p. c.]	Induction period [h]	Prologation by NPA [p. c.]
Without water			
Starch	0	3.2	0
Starch	0.05	7.2	125
Albumin	0	7.2	0
Allbumin	0.05	8.3	15
With water (2 : 1 : 0.1)			
Starch	0	5.7	0
Starch	0.05	5.8	2
Albumin	0	5.4	0
Albumin	0.05	6.0	11

Conclusions

NPA acts as a moderately active antioxidant in polyunsaturated edible oils at relatively low temperatures



a – stabilized with 0.02% NPA; b – without NPA

Fig. 7. Example of reaction course of stabilized rapeseed oil in mixture with starch (1 : 2 m/m), measured in the Oxipres apparatus

(40–60 °C), so that it would show antioxidant activity in storage conditions. The mechanism of inhibitory activity is particularly in decreasing the peroxide value.

The activity of NPA is, however, very low at temperatures of 100 °C and higher, i.e. it would be practically inefficient under baking and deep frying conditions.

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Souhrn

KOGA H., TOKUYAMA T., SAKURAI H., RÉBLOVÁ Z., TROJÁKOVÁ L., NGUYEN H. T. T., POKORNÝ J. (1999): **Antioxidační účinnost N-(2-pyron-3-yl)-α-L-aspartyl-L-fenylalaninmethylesteru.** Czech J. Food Sci., **17**: 41–48.

Při skladování nebo záhřevu potravin nebo nápojů obsahujících kyselinu askorbovou a Aspartam probíhají Maillardovy reakce a hlavním produktem je N-(2-pyron-3-yl)-α-L-aspartyl-L-fenylalaninmethylester. Tato látka vykazuje antioxidační účinnost, která se více projevuje snížením rychlosti hromadění hydroperoxidu v tuku než prodloužením indukční periody. Aktivita α-tokoferolu byla při 60 °C vyšší v nerafinovaném podzemnicovém oleji, projevovala se také při 97 °C, avšak v rafinovaném řepkovém oleji byla aktivita nízká i při 40 °C. S rostoucí teplotou klesala antioxidační aktivita jak v podzemnicovém oleji, tak v řepkovém oleji. Při teplotách smažení již byla nepatrná. Mírný synergistický účinek byl zjištěn mezi pyronem a *terc.* butylhydroxytoluenem a *terc.* butylhydroxyanisolem. V přítomnosti pyronu se mírně zvýšila stabilita směsi oleje s vaječným albuminem a se škrobem, ale účinnost se ztratila v přítomnosti vody. Rozklad pyronu během záhřevu mohl přispět k tmavnutí olejové frakce ve směsi.

Klíčová slova: antioxidační aktivita; kyselina askorbová; Aspartam; hnědnutí; hydroperoxidy; podzemnicový olej; N-(2-pyron-3-yl)aspartylfenylalaninmetylester; řepkový olej

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Antimicrobial Effect of Monolauroylglycerol and Lauric Acid in a Model Emulsion System*

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Abstract

PLOCKOVÁ M., FILIP V., KUKAČKOVÁ O., ŠMIDRKAL J., ŘIHÁKOVÁ Z. (1999): **Antimicrobial effect of monolauroylglycerol and lauric acid in a model emulsion system.** Czech J. Food Sci., **17**: 49–54.

Antimicrobial effectiveness of monolauroyglycerol (monolaurin) and lauric acid against *Bacillus subtilis* LCC 666, *Escherichia coli* DBM 3104 and *Penicillium expansum* DBM 4061 were studied using two agar diffusion assays and confirmed by the testing of the surviving microbial cells in water-in-oil emulsion with monolauroylglycerol and lauric acid. By using agar spot on lawn diffusion assay, which was more sensitive than the agar well diffusion assay, the values of MIC of monolauroylglycerol (MIC_{MLG}) and lauric acid (MIC_{LA}) for *Bacillus subtilis* LCC 666 M_{MLG} = 50 µg/ml, MIC_{LA} = 50 µg/ml, for *Escherichia coli* DBM 3104 MIC_{MLG} = 500 µg/ml, MIC_{LA} = 5 000 µg/ml and for *Penicillium expansum* DBM 4061 MIC_{MLG} = 50 µg/ml, MIC_{LA} = 500 µg/ml were evaluated. Monolauroyglygerol (0.5% w/w) and of lauric acid (0.5 % w/w) were added into the water-in-oil emulsion system. These concentrations completely inhibited 1.10^3 cfu/g of *Bacillus subtilis* LCC 666 during 14 days of storage at 23 °C, decreased 1.10^4 cfu/g of *Escherichia coli* DBM 3104 by 1 log cycle during 28 days of storage at 23 °C and had no evident effect on *Penicillium expansum* DBM 4061 during 28 days of storage at 23 °C.

Key words: *Bacillus subtilis*; *Escherichia coli*; inhibition of growth; lauric acid; monolauroylglycerol; *Penicilium expansum*

A high microbiological standard of food products can be achieved by following the Good Manufacturing Practice and carefully selecting the quality of raw materials. In addition, the conditions for the survival and growth of the present microorganisms ought to be taken into account with respect to the chemical composition, structure, way of storage and distribution of each food product.

For the water-in-oil emulsions the following inner factors are important for the survival and growth of microorganisms: chemical composition of the aqueous phase with respect to its pH and water activity (BROCKLEHURST *et al.* 1993), the size and distribution of water droplets (VERRIPS & ZAALBERG 1980), oxygen contained within the oil phase (BROCKLEHURST *et al.* 1993) and the possible interactions between the aqueous and oil phase (BROCKLEHURST *et al.* 19931; ROBINS *et al.* 1994). Outside factors such as temperature (VERRIPS & ZAALBERG 1980) are also important. In water-in-oil systems, it is the aqueus phase where the microorganisms are growing. The concentration of carbon and nitrogen sources, the concentration of end-products of metabolism and the concentration of antimicrobially active substances such as organic acids, sodium chloride and chemical preservatives in the aqueous phase have a direct influence on the growth of microorganisms (BROCKLEHURST *et al.* 1993).

At this time the use of chemical preservatives such as benzoic and sorbic acids and their salts is limited. The new way of ensuring food safety and improving the quality of heterogeneous food systems seems to be the use of the derivatives of fatty acids possesing both antimicrobial and emulsifying activity. Monolauroylglycerol (hydrophylic – lipophylic balance – HLB 6.6) (JOHN & MCBAIN 1948) and lauric acid (HLB 3.9 at pH 4) (DAVIES 1957) dissolved mainly in the oil phase were tested for their ability to suppress the growth of microorganisms in a model water-in-oil emulsion system.

It was proved in the past that lauric acid derivatives are effective mainly against Gram-positive bacteria (e.g. *Bacillus* sp., *Streptococcus* sp., *Micrococcus* sp.) (KABARA 1993; RAZAVI-ROHANI & GRIFFITHS 1994), lesser effective against moulds and yeasts (KATO & SHISABAKI 1975) and minimally effective against Gram-negative bacteria (KABARA 1993; RAZAVI-ROHANI & GRIFFITHS 1994). Monolauroyglycerol, D-laurate A, T-laurate A and lauric acid were proved to suppress the mould growth on Malt Extract Agar (PLOCKOVÁ *et al.* 1999). By that time mono-

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lauroyglycerol alone or in combination with organic acids, sodium chloride or nisine was succesfully used in meat products (OH DEOG-HWAN & MARSHAL, 1994; BAKER *et al.* 1982; Bell & DE LACY 1987), bakery products (STILLMUNKES *et al.* 1993), cheeses (BAUTISTA *et al.* 1993) and low fat margarines (STILLMUNKES *et al.* 1993). In spite of this the detailed mechanism of action of monolauroyglycerol as a typical surfactant against microorganisms present in the aqueous phase of emulsion systems has not been explained so far.

The aim of our work was to evaluate the MIC of monolauroylglycerol and lauric acid against *Escherichia coli* DBM 3104, *Bacillus subtilis* LCC 666 and *Penicillium expansum* DBM 4061. Further we tried to evaluate the effect of the addition of monolauroyglycerol and lauric acid on the growth rate of the above mentioned microorganisms in a model emulsion system.

MATERIAL A METHODS

Used Strains and their Cultivation Conditions

Strains of microorganisms used in this study were *Escherichia coli* DBM 3104 and *Penicillium expansum* DBM 4061 (from the Collection of Microorganisms of the Department of Biochemistry and Microbiology, ICT Prague, Czech Republic) and *Bacillus subtilis* LCC 666 (from the Culture Collection of Dairy Microorganisms Laktoflora Prague, Czech Republic). Both bacterial strains were maintained on a slant Nutrient Agar (Oxoid, UK) and subcultured once a week at 37 °C for 24 hrs. The mould strain was maintained on the slope Malt Extract Agar (Oxoid, UK) and subcultured once a month at room temperature for 5 days. Before each test the bacterial strains were transferred into the Nutrient Broth and freshly subcultured by use of 1% inoculum and cultivation at 37 °C for 3 hrs. The suspension of mould strain spores and hyphae were prepared by washing the slope agar of the freshly transferred culture with 5 ml of sterile saline with Tween 80.

Used Antimicrobial Agents

Moolauroylglycerol was synthetized at the Department of Dairy and Fat Technology, ICT Prague, Czech Republic (purity 99.9% w/w, lauric acid content 97% w/w). Lauric acid was obtained from Henkel KGaA, Germany (purity 97% w/w). Stock solutions were prepared by dissolving each substance in ethanol (50% v/v).

Determination of Minimal Inhibitory Concentration (MIC)

For the determination of MIC of monolauroylglycerol and lauric acid against *Escherichia coli* DBM 3104, *Bacillus subtilis* LCC 666 and *Penicillium expansum* DBM 4061 two agar diffusion methods were used – the agar spot on lawn diffusion method and the agar well diffusion method. Both methods were compared. The agar well diffusion method was carried out in the following way: 1 ml of the suspension from each test microorganism (for bacterial strains of density corresponding to $A_{615} = 0.2$, for mold strain to $A_{615} = 0.1$) 20 ml of melted agar (Nutrient Agar with 1.5% (w/w) agar for bacterial strains, Malt Extract Agar for the mould strain) was pipetted into Petri dishes and mixed thoroughly. 20 µl of solution from each antimicrobial substance at concentrations of 0, 50, 100, 500 µg/ml, 1, 5, 10 mg/ml were pipetted into the wells (6 mm in diameter) made by sterile cork borer in the centre of solidified agar. All plates were preincubated at 20 °C for 16 hrs. The plates with bacterial test strains were then incubated at 37 °C for 24 hrs with the mould test strain at 23 °C for 5 days. After this time the zones of growth inhibition were detected. The agar spot on lawn diffusion method was carried out in the following manner: 20 ml of melted agar (Nutrient Agar or Malt Extract Agar with 0.7% w/w agar) was pipetted into Petri dish. 0.2 ml of the suspension from each test microorganism ($A_{615} = 0.2$ or $A_{615} = 0.1$) was spread onto the agar surface. 10 ml of solution of antimicrobial agent at concentrations of 0, 50, 100, 500 mg/ml, 1, 5, 10 mg/ml were spotted into Petri dishes. The Petri dishes were preincubated at 20 °C for 16 hrs and then at 37 °C for 24 hrs or at 23 °C for 5 days. The diameters of the clear zones were measured after the cultivation. The presented results are the average values from two trials.

Preparation of the Emulsions and the Conditions of their Storage

The water phase of the emulsion was prepared for 1 kg of model emulsion by dissolving 1.3 g of NaCl and 5.3 g of powdered sweet whey (JČM, a.s., Czech Republic) in 260.8 g of tap water. It was heated for 60 min at 90 °C and after it cooled the pH value was adjusted to 6.45. The suspension of each tested microorganism was added into the sterile water phase at the following amounts: 1.10^4 cfu *Escherichia coli* DBM 3104/g, 1.10^3 cfu *Bacillus subtilis* LCC 666/g, 1.10^4 cfu *Penicillium expansum* DBM 4061/g. The oil phase was prepared for 1 kg of the model emulsion by combining the base of oils for soft margarine with SFC profile: $SFC_{10\,°C} = 25\%$, $SFC_{20\,°C} = 13.5\%$, $SFC_{30\,°C} = 6.4\%$, $SFC_{35\,°C} = 4.4\%$, $SFC_{40°C} = 2.4\%$ (708.2 g), monoglyceride emulsifier (3.9 g), beta-carotene (0.012 g), vitamin A (0.09 g) and vitamin E (0.2 g). Monolauroyglycerol and lauric acid, each at concentrations of 0.5% (w/w), were added straight into the oil phase and the mixture was heated at 62 °C. Emulsification was realized by using a special laboratory apparatus consisting of a sterile planetary stirrer and a cooling apparatus at 42 °C for 5 min at 1200 r.p.m. The emulsion was stored at 23 °C for 4 weeks and tested weekly. The presented results are the average values from two trials.

Evaluation of cfu/g of Added Microorganisms in Model Emulsions

During the 4 week storage of emulsions the number of each inoculated microorganism were evaluated weekly.

Table 1. Minimal inhibition concentrations

	Agar well diffusion assay		Agar spot on lawn diffusion assay	
	MLG [mg/ml]	LA [mg/ml]	MLG [mg/ml]	LA [mg/ml]
Bacillus subtilis LCC 666	500	500	50	50
Escherichia coli DBM 3104	5 000	10 000	500	5 000
Penicillium expansum DBM 4061	5 000	5 000	50	500

MLG – monolauroylglycerol
LA – lauric acid

Average samples for *Escherichia coli* DBM 3104 and *Bacillus subtilis* LCC 666 evaluations were prepared each as an average one by sampling the mass from five different places of emulsion. Separate samples for evaluation of cfu of *Penicillium expansum* DBM 4061 were prepared from the surface (3mm depth. layer) and the inner part of the emulsion (each by sampling from five different places). Cfu/g of *Bacillus subtilis* LCC 666 was evaluated by the use of TPC Agar (Oxoid, UK) – after 72 hrs at 37 °C, *Escherichia coli* DBM 3104 by the use of VRBL Agar (Milcom, Czech Republic) – after 48 hrs at 37 °C and *Penicillium expansum* DBM 4061 by the use of GYCH (glucose, yeast extract, chloramphenicol) Agar (Milcom, Czech Republic) after 5 days at 23 °C.

RESULTS AND DISCUSSION

Escherichia coli, a typical Gram-negative bacterium which indicates poor cleaning conditions in food processing plants, *Bacillus subtilis*, a Gram-positive sporeforming bacterium occurring the in air or on dust particles, and *Penicillium expansum,* whose vegetative spores are also present in the air or soil. All of raamed microorganisms can be isolated from the air and surfaces of the food processing plant equipment (HAYES 1995) were chosen as a model microorganisms in this work.

First MIC of monolauroyglycerol and lauric acid against three model test microorganisms were measured by the use of agar spot on lawn diffusion method and agar well diffusion method. The possible antimicrobial effect of ethanol (solvent of monolauroyglycerol and lauric acid) was eliminated by the use of a blank trial with ethanol alone. The results are presented in Table1.

Agar spot on lawn diffusion assay was found to be more sensitive than the well agar diffusion assay. Monolauroylglycerol showed in most cases higher antimicrobial effects compared with lauric acid. The most sensitive strain against both monolauroyglycerol and lauric acid was *Bacillus subtilis* LCC 666 followed by *Penicillium expansum* DBM 4061 and *Escherichia coli* DBM 3104. This fully agrees with previously published results (KABARA 1993; RAZAVI-ROHANI & GRIFFITHS 1994]. In the case of *Penicillium expansum* DBM 4061 not only suppression of mycelial growth but also suppression of spore forming, visible as a change in colour (white yellow instead of dark green), was observed.

The samples of water-in-oil model emulsions system alone, with 0.5% (w/w) monolauroylglycerol or with 0.5% (w/w) lauric acid inoculated by *Escherichia coli* DBM 3104 (1.10^4 cfu/g) or *Bacillus subtilis* LCC 666 (1.10^3 cfu/g) or *Penicillium expansum* DBM 4061 (1.10^4 cfu/g) were stored at 23 °C for 28 days. The numbers of cells surviving in the

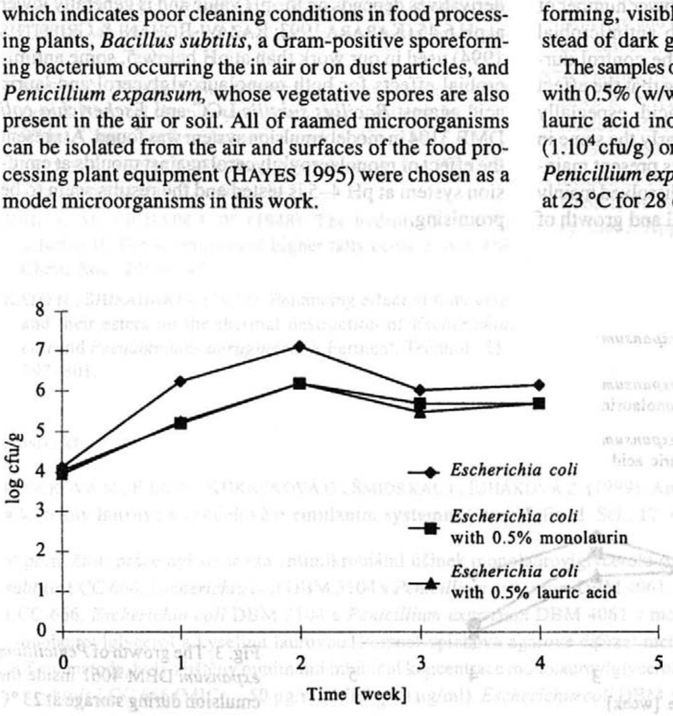

Fig. 1. The growth of *Escherichia coli* DBM 3104 in emulsion during storage at 23 °C

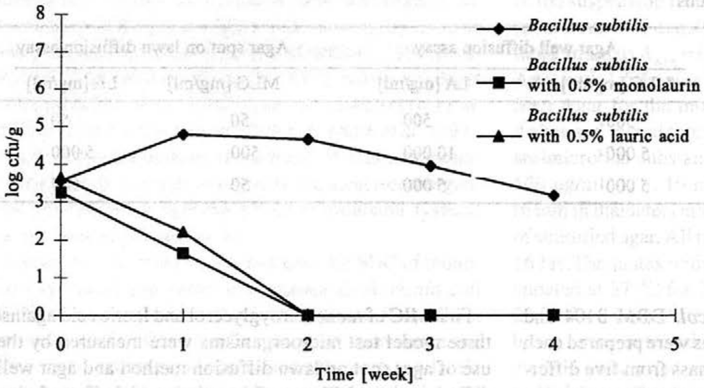

Fig. 2. The growth of *Bacillus subtilis* LCC 666 in emulsion during storage at 23 °C

model emulsion system are presented in Fig. 1–4. The pH value of emulsion 6.45 was used with the aim to eliminate the effect of low pH on the growth of microorganisms.

In this experiment the highest sensitivity of *Bacillus subtilis* LCC 666 against tested agents was acknowledged. As it is shown in Fig. 1 the number of cfu of *Bacillus subtilis* LCC 666 decreased by 3 log cycles in the presence of 0.5% (w/w) monolauroylglycerol and by 2 log cycles in the presence of 0.5% (w/w) lauric acid during the first week of storage. During the second week of storage viable cells of *Bacillus subtilis* LCC 666 disappeared totally in the presence of both monolauroyglycerol and lauric acid. During the storage of emulsion with *Escherichia coli* DBM 3104 (Fig. 2) about 1 log cycle lower number of cfu was found in the emulsions in which antimicrobial compounds were added compared with the control during the whole storage time. It is interesting that the effect of both monolauroyglycerol and lauric acid, especially against *Bacillus subtilis* LCC 666, was nearly the same in spite of the fact that monolauroyglycerol is present mainly in water-oil interphase and lauric acid is dissolved mainly in oil phase while the place of the survival and growth of bacteria is aqueous phase.

Water phase of the emulsions was inoculated with *Penicillium expansum* DBM 4061. After solidification the samples were taken both from the inner part and from the outer part with regard to the better growth conditions of moulds on the surface of emulsions. The results for both parts of the emulsion are presented in Fig. 3–4. The difference in the inner and the outer part of the emulsion was about 2 log cycles in favour of the outer part at the end of storage. The suppressive effect of adding monolauroylglycerol and lauric acid to the growth of *Penicillium expansum* DBM 4061 in the inner and outer part of the emulsion was not proved.

Although the antimicrobial effectiveness of lauric acid derivatives depends on the pH value and is generally lower at pH 6.45 (KABARA 1993; RAZAVI-ROHANI & GRIFFITHS 1994) used in our work than at pH below 5, some antimicrobial effects for both monolauroylglycerol and lauric acid against *Bacillus subtilis* LCC and *Escherichia coli* DMB 3104 in model emulsion system was found. At present the effect of monolauroylglycerol against moulds at emulsion system at pH 4–5 is tested and the results seem to be promising.

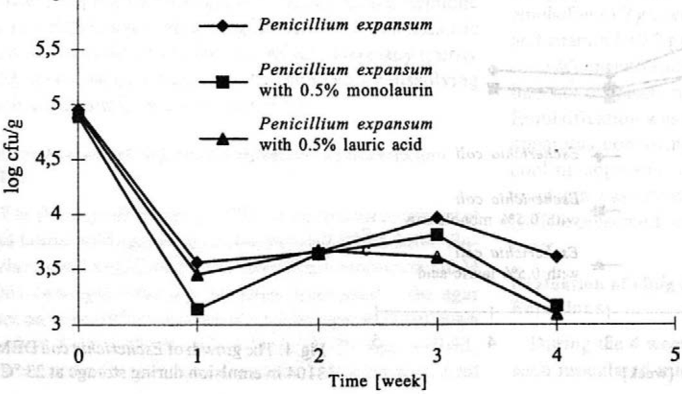

Fig. 3. The growth of *Penicillium expansum* DBM 4061 inside the emulsion during storage at 23 °C

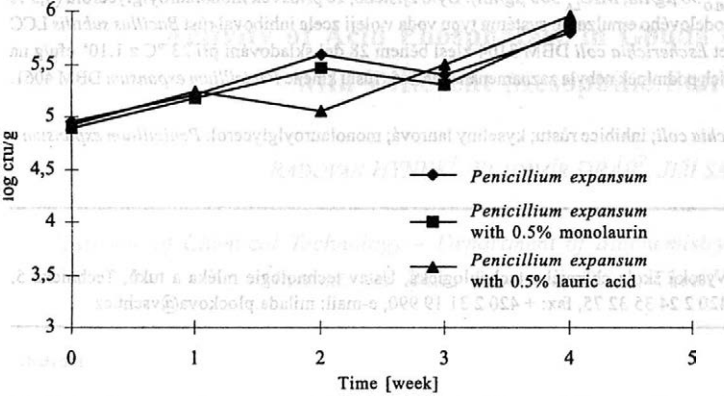

Fig. 4. The growth of *Penicillium expansum* DBM 4061 on emulsion surface during storage at 23 °C

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Abstrakt

PLOCKOVÁ M., FILIP V., KUKAČKOVÁ O., ŠMIDRKAL J., ŘIHÁKOVÁ Z. (1999): **Antimikrobiální účinek monolauroylglycerolu a kyseliny laurové v modelovém emulzním systému**. Czech J. Food. Sci., **17**: 49–54.

V první části práce byl sledován antimikrobiální účinek monolauroylglycerolu (monolaurinu) a kyseliny laurové vůči *Bacillus subtilis* LCC 666, *Escherichia coli* DBM 3104 a *Penicillium expansum* DBM 4061. Dále bylo testováno přežívání *Bacillus subtilis* LCC 666, *Escherichia coli* DBM 3104 a *Penicillium expansum* DBM 4061 v modelovém emulzním systému, který obsahoval monolauroylglycerol a kyselinu laurovou. Pomocí vpichové agarové difúzní metody, která byla citlivější než dírková agarová difúzní metoda, byly zjištěny minimální inhibiční koncentrace monolauroylglycerolu (MIC_{MLG}) a kyseliny laurové (MIC_{LA}): *Bacillus subtilis* LCC 666 (MIC_{MLG} 50 μg/ml, MIC_{LA} 50 μg/ml), *Escherichia coli* DBM 3104 (MIC_{MLG} 500 μg/ml, MIC_{LA} 5 000 μg/ml), *Penicillium expansum* DBM 4061 (MIC_{MLG} 50 µg/ml, MIC_{LA} 500 µg/ml). Bylo zjištěno, že přídavek monolauroylglycerolu (0,5 % hm.) a kyseliny laurové (0,5 % hm.) do modelového emulzního systému typu voda v oleji zcela inhiboval růst *Bacillus subtilis* LCC 666 za 14 dní skladování při 23 °C. Počet *Escherichia coli* DBM 3104 klesl během 28 dní skladování při 23 °C z 1.10^4 cfu/g na 1.10^3 cfu/g. Při použití stejných skladovacích podmínek nebyla zaznamenána inhibice růstu kmene *Penicillium expansum* DBM 4061.

Klíčová slova: *Bacillus subtilis*; *Escherichia coli*; inhibice růstu; kyseliny laurová; monolauroylglycerol; *Penicillium expansum*

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Activity of Acid Phosphatase in Gouda Cheese Made with Different Mesophilic Starters

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Abstract

HYNEK R., DRÁB V., SAJDOK J. (1999): **Activity of acid phosphatase in Gouda cheese made with different mesophilic starters**. Czech J. Food Sci., **17**: 55–60.

Activity of acid phosphatase during 120 days of ripening of Gouda cheese made with five different mesophilic starters was measured. Basic parameters as well as final sensory properties of cheeses were also evaluated. Reversed-phase HPLC was used for separation of peptides. The cheeses made with starter 973 had the lowest activity of acid phosphatase, whereas the cheeses made with starter F24 had the highest activity. It seems that differences in the activity of acid phosphatase have influence on the nature of casein breakdown products and on sensory properties of cheeses. Obtained results of RP-HPLC showed the highest concentration of hydrophobic peptides in cheese samples made with starter 973. Taste of these samples was very bitter. It is probable that low activity of acid phosphatase can lead to accumulation of hydrophobic, often bitter, peptides which are undesirable for final sensory properties of cheese.

Key words: Gouda cheese; mesophilic starters; acid phosphatase activity

Casein is a major phosphoprotein which is present in milk. This phosphoprotein is cleaved by proteolytic enzymes during cheese ripening into a number of peptide fragments. Peptide fragments which are phosphorylated seem to have different significant properties. Peptides containing cluster sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- have been shown to stabilize amorphous calcium phosphate at neutral and alkaline pH and to be anticariogenic in various *in vitro* experiments (REEVES & LATOUR 1958; REYNOLDS 1987, 1991). Other studies have shown that casein phosphopeptides are formed *in vivo* by normal digestion of casein and as they are relatively resistant to further proteolytic degradation, they can accumulate in the distal part of the small intestine. It has been proposed that this accumulation together with the peptide ability of forming soluble complexes with calcium phosphate is responsible for the enhanced intestinal calcium absorption that has been observed even in the case of vitamin D-deficiency (MEISEL & FRISTER 1988; MYKKANEN& WASSERMAN 1980; NAITO *et al.* 1972; NAITO & SUZUKI 1974; SATO *et al.* 1986). Casein phosphopeptide-metal ion complexes, therefore, have potential as dietetic supplements to increase the bioavailability of calcium, iron and other essential metal ions. Casein phosphopeptides were isolated from water extract of cheese (ROUDOT *et al.* 1994).

Activity of acid phosphatase during production of cheese seems to influence the degree of phosphorylation of casein and peptides which are products of its enzymatic hydrolysis (ADDEO *et al.* 1992). Low activity of acid phosphatase causes a higher degree of phosphorylation of casein and thus could cause inhibition of complete proteolytical cleavage of this protein (DULLEY & KITCHEN 1973). It means that lower activity of acid phosphatase causes a higher degree of phosphorylation of peptides, which are created by proteolytical cleavage of casein during cheese maturation. On the other hand, higher activity of acid phosphatase causes a lower degree of phosphorylation of the mentioned peptides. Some casein phosphopeptides are not cleaved into smaller peptide fragments, which can lead to the accumulation of longer peptides which have negative influence on final sensory properties of cheese. It seems that the activity of acid phosphatase may play an important role during cheese production.

Activity of acid phosphatase, during ripening of Gouda cheese made with five different mesophilic starters, was measured in this study. Acid phosphatase activity was compared with sensory properties of cheese as well as with a chromatographic profile of proteolytical digest (mixture of peptides) after 120 days of maturation.

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MATERIAL AND METHODS

Cheese Production and Sampling

In pilot scale using 200 l vats, a set of four Gouda cheeses was produced by using a standard manufacturing schedule. Five different mesophilic DL-starters from Laktoflora (Prague, Czech Republic) were used. Three replicate series of these cheese making trials were performed. Cheese wheels were ripened at 14 °C under plastic coat Fungus from Lamirsa (Barcelona, Spain). At pre-determinate ages of 5, 15, 30, 60, 90 and 120 days samples were cut from cheese wheel. After removing the rind, the remaining cheese mass was used for analysis.

Determination of Basic Parameters

- dry matter (DM, expressed in g/100g of cheese) was determined by drying for 4 hrs at temperature 102 °C;
- fat (F, expressed in g/100g of cheese) was determined by acidobutyrimetric method according to Van Gulik;
- fat in dry matter (FDM, expressed in g/100g of dry matter);
- NaCl (expressed in g/100g of cheese) was determined by argentometric titration;
- active acidity (pH) was measured by probing a plastic electrode Inlab 427 Mettler-Toledo (Greifensee, Switzerland) into the compressed grated cheese on Hanna HI 8424;
- pH 4.6–soluble nitrogen (NCN), 12% trichloroacetic acid–soluble nitrogen (NPN) and 5% phosphotungstic acid–soluble nitrogen (PTA) were determined from aqueous extract by the Kjeldahl method (SNÁŠELOVÁ *et al.* 1996) on Kjeltec Auto 1030 Analyser from Tecator (Hoganas, Sweden) and were expressed as percentage of total nitrogen.

Cheese Extracts

Aqueous cheese extract for determination of acid phosphatase activity and high performance liquid chromatography (HPLC) analysis of peptides in 70% ethanol was prepared by dilution of 15 g grated cheese with 45 ml of deionized water and subsequent homogenization by Ultra Turrax T25 (Janke & Kunkel, FRG). Homogenization was performed 2 min at 12 500 rpm with 1 min pause after 1 min of homogenization. After homogenization the weight of mixture was adjusted to 80 g by addition of deionized water and the mixture was incubated 30 min at 30 °C. Then the centrifugation was performed for 10 min at 5000 rpm and 4 °C. The fat layer was removed and the aqueous phase was filtered through Filtrak 390 (Filtrak, FRG). The resulting filtrate was used for determination of acid phosphatase activity or 10 ml of filtrate was mixed with 28 ml of 95% ethanol and after 60 min of precipitation at room temperature was centrifuged 10 min at 5000 rpm and clear supernatant was removed by pipette. Obtained extracts were stored at –18 °C until analysis.

Determination of Acid Phosphatase Activity

The modification of Larsen and Parada method was used for determination of acid phosphatase activity (LARSEN & PARADA 1988). Aqueous cheese extract (0.25 ml) was mixed with 0.25 ml of solution of hexahydrate of disodium p-nitrophenylphosphate (0.25 g/100 ml) (Sigma 104 phosphatase substrate) and 0.5 ml of 1M sodium acetate buffer (pH 4.8). After 16 hrs of incubation at 37 °C the reaction was stopped with 1 ml of 12% (m/v) trichloroacetic acid. Precipitated proteins were removed by centrifugation at 5000 rpm for 10 min and 0.7 ml of the supernatant was mixed with 3 ml of 1M NaOH. Absorbance at 405 nm was measured and results were expressed as phosphatase units/g of dry matter (LARSEN & PARADA 1988). The method was preliminarily tested on raw milk and several kinds of cheese. Results for raw cow's milk and blue cheese (Niva) were comparable with results of LARSEN and PARADA (1988). We determined 2.93×10^{-3} and 1.01×10^{-2} phosphatase units/g of dry matter, while LARSEN and PARADA (1988) determined 2.9×10^{-3} and 1.3×10^{-2} phosphatase units/g of dry matter. Phosphatase activity in Gouda cheese was comparable with results for Crema cheese. The amount of liberated *p*-nitrophenol after 60 min was small in water extracts of Gouda cheese and for this reason the original method was modified and time of incubation was extended to 16 hours. Standard solution (10 mmol/ml) of *p*-nitrophenol (Sigma Diagnostics, USA) was used for calibration. Values in Table 2 are averages of two measurements (SD = standard deviation).

Reversed-phase HPLC Separation of Peptides

Peptides contained in ethanol extract were separated on Hewlett-Packard 1090 Series II Liquid Chromatograph on a reversed-phase column (250 × 4 mm I.D.) (OD-584, HP) LiChrospher 100 RP-18 (5 mm). Injection volume was 250 ml, flow-rate 1.0 ml/min. Solvent A = trifluoroacetic acid – water (0.1 : 99.9 v/v), solvent B = solvent A–acetonitrile (20 : 80 v/v). Linear gradient: 100 % A for 7 min, 0–90 % B generated over 53 min. Peptides were detected at 215 nm.

Sensory Evaluation

The texture, body and flavour of the cheese were assessed organoleptically after 120 days of ripening. Five trained panellists graded the cheese properties at a scale 1–5 (1 superb, 2 good, 3 approved, 4 bad, 5 very bad) according to the criteria defined for each cheese variety. Scores presented are means of grades by all panellists.

RESULTS AND DISCUSSION

Five different mesophilic starters were used for experimental production of Gouda cheese. Representative values of obtained results (dry matter, pH, concentration of NaCl, fat in dry matter, NCN, NPN and PTA), as well as result of sensory evaluation after 60, 90 and 120 days of maturation are shown in Table 1. Activities of acid phosphatase in 5 days old cheeses made with different starters are plotted in Fig. 1. Acid phosphatase activities during ripening of cheeses are summarised in Table 2. RP-HPLC (reversed phase HPLC) profiles of ethanol soluble casein breakdown products of samples prepared by all five mesophilic starters after 120 days of cheese maturation are shown in Fig. 2a–e.

The lowest activity of acid phosphatase was obviously found in culture 973 (Fig. 1, Table 2). Sensory evaluation of corresponding sample of cheese after 120 days of maturation was the worst of all samples (degree 4, 4.5, 4). Flavour of these cheeses was bitter and was evaluated as uneatable and thus microbial culture 973 was not recommended for production of Gouda cheese. A relatively high number of peptides, whitch were eluted latter in comparison with Fig. 2b–d and particularly with Fig. 2e can be seen in Fig. 2a. Peptides which show peaks with higher retention times are eluted from reversed-phase column with higher concentrations of acetonitril and we can judge from this fact that they are more hydrophobic. Hydrophobic peptides have negative influence on sensory properties of cheeses because of their bitter taste. The highest concentration of hydrophobic peptides (with retention times higher than 35 min) is apparently in the sample prepared with culture 973 (Fig. 2a) which has the lowest activity of acid phosphatase. We can see the lower concentration of hydrophobic peptides in three samples which were prepared using cultures 933, 16 and 1026 (Fig. 2b–d) with higher activity of acid phosphatase (Table 2 and

Table 1. Basic parametres and sensory properties of Gouda cheese

Starter	Sample	Age [days]	DM [%]	pH	NaCl [%]	FDM [%]	NCN [%]	NPN [%]	PTA [%]	Sensory analysis
973	ND 3/5	5	58.41	5.24	1.66	51.4	6.0	3.1	0.7	
	ND 3/15	15	61.23	5.31	2.08		9.8	4.8	0.8	
	ND 3/30	30	61.93	5.33	2.27	50.9	13.0	7.3	1.5	
	ND 3 /60	60	66.66	5.42	2.39	51.0	16.5	9.5	1.8	4.0
	ND 3/90	90	69.13	5.45	2.54	49.5	20.7	12.3	1.9	4.5
	ND 3/120	120	67.29	5.51	2.65	51.3	23.0	14.0	2.4	4.0
933	ND 4/5	5	55.67	5.37	2.11	44.9	5.9	2.8	0.8	
	ND 4/15	15	58.28	5.51	2.46	42.9	9.5	5.2	1.3	
	ND 4/30	30	60.42	5.49	2.61	44.7	12.8	7.2	1.9	
	ND 4/61	61	64.52	5.56	2.49	44.2	17.8	9.8	3.0	2.5
	ND 4/90	90	62.92	5.60	2.63	45.3	22.8	12.2	4.3	3.0
	ND 4/120	120	64.36	5.59	2.82	46.2	25.9	15.3	4.6	1.5
16	ND 17/6	6	58.10	5.46	1.90	46.5	5.4	3.2	0.8	
	ND 17 /15	15	59.03	5.50	2.16	46.6	7.7	4.6	1.2	
	ND 17/30	30	61.21	5.58	2.63	47.4	13.2	7.4	1.6	
	ND 17/60	60	61.28	5.50	2.60	46.5	17.0	10.1	2.3	2.0
	ND 17/90	90	64.11	5.52	2.78	46.0	21.7	12.3	3.3	4.0
	ND 17/120	120	65.33	5.56	2.91	46.7	22.9	14.2	3.6	2.0
1026	ND 14/5	5	57.53	5.46	2.19	43.5	4.4	2.3	0.6	
	ND 14/15	15	60.02	5.47	2.34	42.5	7.8	4.1	0.8	
	ND 14/30	30	60.26	5.51	2.71	42.3	11.7	6.3	1.0	
	ND 14 /60	60	62.67	5.52	2.61	41.5	15.1	7.7	2.6	1.5
	ND 14/90	90	64.06	5.53	2.52	42.1	18.0	10.1	1.5	2.5
	ND 14/120	120	65.95	5.62	3.18	43.2	21.3	11.4	1.6	1.0
F24	ND 19/5	5	58.50	5.38	2.45	45.3	5.3	3.0	0.8	
	ND 19/15	15	60.37	5.48	2.60	47.2	9.4	4.8	1.2	
	ND 19/30	30	61.31		2.76	45.7	13.0	7.5	1.6	
	ND 19/60	60	61.78	5.48	3.11	46.5	17.8	10.2	2.6	3.0
	ND 19/90	90	63.88	5.54	2.71	45.4	20.6	12.0	2.9	1.8
	ND 19 /120	120	64.39	5.57	3.14	45.0	21.8	14.0	3.5	1.5

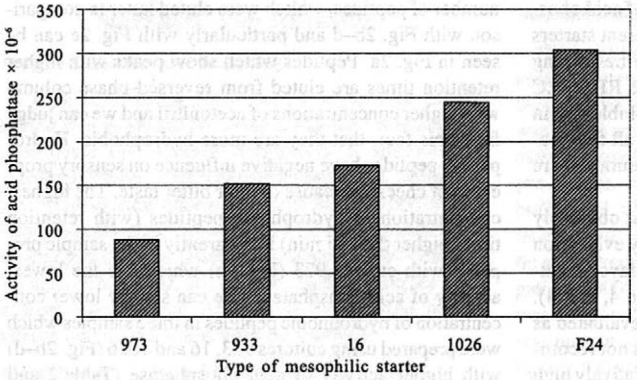
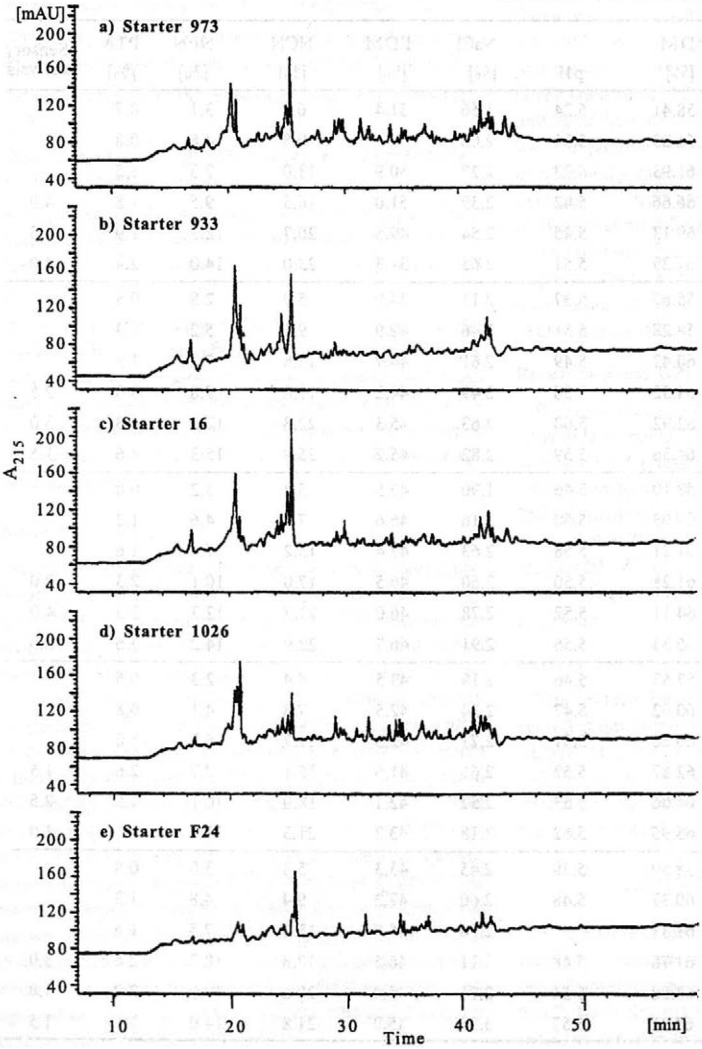


Fig. 1. Activity of acid phosphatase after five days of cheese maturation [units/g of dry matter]



A_{215} – absorbance at 215 nm

For separation conditions see the text

Fig. 2. RP-HPLC profile of casein breakdown products

Table 2. Activity of acid phosphatase (expressed as units/g of dry matter)

Starter	Sample	Age [days]	Acid phosp. activity	SD
973	ND 3/5	5	88.0×10^{-6}	1.8×10^{-6}
	ND 3/15	15	51.3×10^{-6}	2.7×10^{-6}
	ND 3/30	30	32.8×10^{-6}	0.5×10^{-6}
	ND 3 /60	60	46.6×10^{-6}	3.0×10^{-6}
	ND 3/90	90	87.2×10^{-6}	4.5×10^{-6}
	ND 3/120	120	55.6×10^{-6}	3.5×10^{-6}
933	ND 4/5	5	152.2×10^{-6}	1.9×10^{-6}
	ND 4/15	15	130.2×10^{-6}	9.1×10^{-6}
	ND 4/30	30	128.6×10^{-6}	4.7×10^{-6}
	ND 4/61	61	96.3×10^{-6}	4.3×10^{-6}
	ND 4/90	90	86.4×10^{-6}	1.7×10^{-6}
	ND 4/120	120	101.8×10^{-6}	2.7×10^{-6}
16	ND 17/6	6	172.6×10^{-6}	0.6×10^{-6}
	ND 17 /15	15	136.7×10^{-6}	2.4×10^{-6}
	ND 17/30	30	168.7×10^{-6}	1.8×10^{-6}
	ND 17/60	60	232.0×10^{-6}	3.5×10^{-6}
	ND 17/90	90	218.3×10^{-6}	2.3×10^{-6}
	ND 17/120	120	211.4×10^{-6}	6.1×10^{-6}
1026	ND10/5	5	244.5×10^{-6}	6.3×10^{-6}
	ND10/15	15	162.8×10^{-6}	7.1×10^{-6}
	ND10/30	30	149.2×10^{-6}	4.1×10^{-6}
	ND10/60	60	189.0×10^{-6}	6.9×10^{-6}
	ND10/90	90	170.4×10^{-6}	11.7×10^{-6}
	ND10/120	120	160.5×10^{-6}	4.3×10^{-6}
F24	ND 19/5	5	303.8×10^{-6}	14.9×10^{-6}
	ND 19/15	15	268.6×10^{-6}	2.4×10^{-6}
	ND 19/30	30	141.9×10^{-6}	13.3×10^{-6}
	ND 19/60	60	225.9×10^{-6}	16.9×10^{-6}
	ND 19/90	90	217.9×10^{-6}	10.2×10^{-6}
	ND 19 /120	120	280.0×10^{-6}	18.6×10^{-6}

Fig. 1). In a sample which was prepared using culture F 24 with the highest activity of acid phosphatase, the lowest concentration of hydrophobic peptides was found (Fig. 2e).

As previously mentioned, low activity of acid phosphatase in microbial cultures causes a higher degree of phosphorylation of casein and peptides released from its proteolytic cleavage. Higher degree of phosphorylation can inhibit further proteolytic cleavage of peptides. It seems that low activity of acid phosphatase can lead to accumulation of undesirable peptides during cheese maturation. We would like to compare changes in content of individual phosphopeptides in the samples which had considerably different activity of acid phosphatase for confirmation of this assumption.

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Souhrn

HYNEK R., DRÁB V., SAJDOK J. (1999): **Aktivita kyselé fosfatázy v sýrech typu Gouda vyrobených různými mezofilními kulturami.** Czech J. Food Sci., **17**: 55–60.

Aktivita kyselé fosfatasy byla měřena během 120 dnů zrání v sýrech typu Gouda vyrobených s použitím pěti různých mezofilních kultur. Sledovány byly rovněž základní parametry a výsledné senzorické vlastnosti sýrů. Peptidy vzniklé proteolytickým štěpením byly separovány pomocí chromatografie na reverzní fázi (RP-HPLC). Sýry vyrobené pomocí kultury 973 měly nejnižší aktivitu kyselé fosfatasy, zatímco v sýrech vyrobených s použitím kultury F 24 byla zjištěna nejvyšší aktivita kyselé fosfatasy. Zdá se, že rozdíly v aktivitě kyselé fosfatasy ovlivňují enzymový rozklad kaseinu a senzorické vlastnosti sýrů. Pomocí RP-HPLC (HPLC na reverzní fázi) byly zjištěny nejvyšší koncentrace hydrofobních peptidů ve vzorcích sýrů vyrobených s použitím kultury 973. Chuť těchto vzorků byla velmi hořká. Hořká chuť je pravděpodobně způsobena nízkou aktivitou kyselé fosfatasy, která vede k hromadění hydrofobních, často hořkých peptidů, které mají nežádoucí vliv na výsledné senzorické vlastnosti sýrů.

Klíčová slova: sýr Gouda; mezofilní kultury; aktivita kyselé fosfatasy

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Phytoestrogens in Soy Foods: Determination of Daidzein and Genistein by Capillary Electrophoresis

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Abstract

VÄNTTINEN K., MORAVCOVÁ J. (1999): **Phytoestrogens in soy foods: determination of daidzein and genistein by capillary electrophoresis**. Czech J. Food Sci., **17**: 61–67.

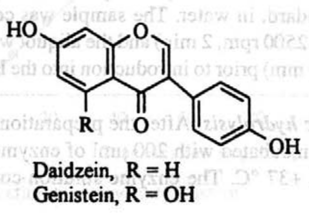
A fast and selective capillary electrophoresis method (CE) was developed for the determination of the major dietary phytoestrogens. Isoflavones, daidzein and genistein, were separated on an uncoated fused-silica column using 200mM borate buffer and diode array detection at 254 and 268 nm, respectively. The response was linear from 5 to 100 mg/dm^3; the minimum detectable limit was 0.4 mg/dm^3 for both analytes. The relative response factors were 0.519 and 0.755 for daidzein and genistein, respectively, using *p*-nitrophenol as internal standard. Solvent extraction was chosen for isolation of phytoestrogens from soy products and enzymatic hydrolysis was used for the aglycone production. This method was applied to analyze soy flour and tofu and was evaluated for precision. The CE method is useful for routine analyses due to its speed and simple sample handling.

Key words: phytoestrogens; food; determination; isoflavones; soy; daidzein; genistein; capillary electrophoresis

Phytoestrogens, the phenolic compounds synthesised in all higher plants, can be regarded as part of a broader class of antimicrobial molecules known as phytoalexins. Generally, phytoalexins have been defined as substances formed by host tissue in response to injury, infectious agents or other physiological stimuli, accumulating to levels which can inhibit the growth of microorganisms (KUC 1972). The classical definition of phytoestrogens refers to compounds that exert estrogenic effects on the central nervous system, induce estrus, and stimulate growth of the genital tract of female animals (LIEBERMAN 1996).

Phytoestrogens may alter sex hormone production and by biochemical mechanisms modulate their metabolism or action at the cellular level. They influence intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation and differentiation, angiogenesis, calcium transport, Na$^+$/K$^+$ ATPase, vascular smooth muscle cells and due to antioxidant activity also lipid oxidation (ADLERCREUTZ 1995; ADLERCREUTZ & MAZUR 1997). Studies in humans, animals, and cell culture systems suggest that the intake of dietary phytoestrogens plays an important role in prevention of menopause symptoms, osteoporosis, cancer, and heart disease (BRANDI 1997). Simultaneously, a few potentially harmful effects have been shown as well. Isoflavones (SHUTT *et al.* 1967) were found to be responsible for the specific infertility problems of sheep feeding on forage plants such as subterranean clover (*Trifolium subterraneum* L.). Moreover, their negative role cannot be excluded in the early stage of carcinogenesis (MESSINA *et al.* 1994). The nature of the effects may vary as a result of a number of factors, including exposure dose, differences between compounds and their metabolites, presence of other dietary compounds, or other yet unknown factors.

Because of their properties, isoflavones are considered to be phytoestrogens. The isoflavones are narrowly distributed taxonomically, being almost entirely restricted to the subfamily *Papilionoideae* of *Leguminosae*, rarely they occur in other families, such as *Rosaceae*, *Iridaceae* and *Compositae* (ADLERCREUTZ & MAZUR 1997). The isoflavones occur in plants mainly as β-glycosides, 6′′-*O*-malonylglycosides or 6′′-*O*-acetylglycosides, small amounts of them are present also in the free aglycone form. Glucose is the sugar most commonly involved. Dietary exposure of isoflavones to humans is generally attributed to soy food consumption. Daidzein and genistein are the



Daidzein, R = H
Genistein, R = OH

major isoflavones found in this foods the content of which is determined by the processing used (BARNES *et al.* 1994; COWARD *et al.* 1994).

Suitable analytical methods are therefore required for the detection of this class of compounds in food and plants. The reversed phase HPLC has been frequently described for the determination of both forms of phytoestrogens (CARLSON & DOLPHIN 1980; MURPHY 1981; KITADA *et al.* 1986; LUNDH *et al.* 1988; BARBUCH *et al.* 1989; WANG *et al.* 1990; FRANKE & CUSTER 1994, 1996; COWARD *et al.* 1996; FRANKE *et al.* 1998; GAMACHE & ACWORTH 1998). The free aglycone can be alternatively analyzed after derivatization by a tandem GC/MS (ADLERCREUTZ *et al.* 1991, 1993; MAZUR *et al.* 1996, 1998). The radioimmunoassay for daidzein and genistein suitable for sensitive detection in human body fluids as well as in beer has been also reported (HAMPL *et al.* 1998; LAPČÍK *et al.* 1998). However, analysis of these compounds from plant extracts is difficult, often requiring tedious and time-consuming sample handling and there are more opportunities for variability and errors to creep in. Finally, the high performance capillary electrophoresis (HPCE) has been introduced into analytical methods of daidzein and genistein (SHIHABI *et al.* 1994; ARAMENDIA *et al.* 1995). Because of its speed and very high separation efficiency, HPCE is now the most rapidly expanding analytical technique. Furthermore, in HPCE there has been considerable interest in performing single-step analyses, with a simple sample preparation technique.

In this study, we present a simple HPCE technique to determine daidzein and genistein in soy foods.

MATERIAL AND METHODS

Sodium chloride, calcium oxide, sodium borate decahydrate and boric acid were purchased from Lachema (Czech Republic), daidzein and genistein from Sigma-Aldrich Chemie (Germany), β-glucuronidase from Boehringer Mannheim (Germany) and trifluoracetic acid (99.5%) from Fluka AG (Germany). Acetonitrile and *p*-nitrophenol were obtained from Merck (Germany). Food samples, tofu and soy flour, were bought from local shops in Prague.

Sample Preparation for HPCE

Extraction. Food material was dried and homogenized to a fine powder. The samples (30 mg) were vortex-mixed (Ika-Vibrax) in a centrifuge tube with 1 ml of extraction solution for 1 h. The extraction solution consisted of 66% acetonitrile, 0.4% NaCl and *p*-nitrophenol (30 mg/l), as an internal standard, in water. The sample was centrifuged (MPW-310, 2500 rpm, 2 min) and the aliquot was filtered (Anotop, 0.1 mm) prior to introduction into the HPCE column.

Enzymatic hydrolysis. After the preparation 200 µl of aliquot was incubated with 200 µml of enzyme solution overnight at +37 °C. The enzyme solution consisted of 200 µl of β-glucuronidase and 2 ml of borate buffer pH 4.0. Following the incubation, the mixture was filtrated by membrane filter (Anotop, 0.1 µm) and introduced into the instrument.

Acid hydrolysis. After the preparation 200 µl of aliquot was incubated with 2 µl of trifluoracetic acid for 1 h in hot water bath (+95 °C). Following incubation, 400 µl of extracting solution without internal standard was added to dry sample and the mixture was neutralised by CaO to neutral pH checked by indicator paper. The mixture was filtered by Anotop and introduced into HPCE.

Conditions for HPCE

The separation buffer consisted of 200mM sodium borate, the pH of the buffer was adjusted to 8.6 by adding of boric acid. A HP ^{3D}CE instrument, uncoated capillary (50 µm I.D × 50 cm to the detector window, 56 cm total length) and HP ChemStation were used. Sample injection was performed in pressure mode (150 mbar.s). Prior to injection of an acetonitrile-water solution of extracts or aqueous solution of standards, the capillary was purged with 1M sodium hydroxide (1 min), water (1 min) and filled with buffer (3 min). The UV detection was set at 254, 268 and 400 nm for daidzein, genistein and *p*-nitrophenol, respectively. The applied purging vacuum pressure was 2 kPa. The separation potential was 10 kV (positive polarity), which corresponds to the electric current of 60 µA. The column temperature was kept at 25 °C. The running time was 30 min for all solutions.

Statistical Evaluation

The analytical data were evaluated by means of the correlation analysis. The standard deviation and relative standard deviation were calculated according to Equations 1 and 2, respectively.

$$SD = \left[\frac{\Sigma\left(x_i - x_{av}\right)^2}{n}\right]^{1/2} \qquad [1]$$

$$RSD = \frac{100\ SD}{x_{av}} \qquad [2]$$

where: x_i – actual concentration of phytoestrogen
x_{av} – average concentration of phytoestrogen
n = 10

RESULTS AND DISCUSSION

The experimental conditions were set analogously to the conditions published for determination of daidzein and genistein in soybean seeds (SHIHABI *et al.* 1994). The migration time in borate buffer (pH 8.6) was 13.52 for daidzein and 13.80 for genistein (Fig. 1). Using a diode array detector, it was possible to monitor each analyte at the wavelength in maximum of its UV band. Thus, daid-

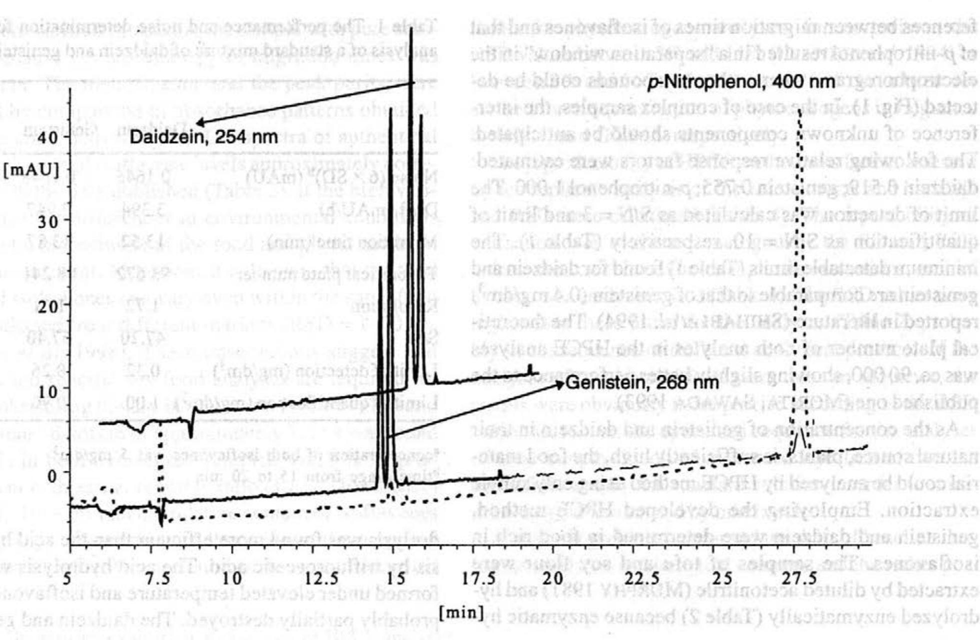


Fig. 1. Electropherogram of a standard mixture (concentration 30 mg/dm³ of each compound)

zein was detected at 254 nm, genistein at 268 nm, and *p*-nitrophenol (internal standard, IS) at 400 nm. In order to check the reproducibility of the experimental data, the standard deviation (SD) of fluctuation of migration times (0.34 min) and relative standard deviations (RSD) of 2.50 and 2.48% for daidzein and genistein, respectively, were estimated from ten repeated injections of standard solutions. The linearity range was determined using both genistein and daidzein. The solutions of genistein and daidzein at a concentration of 100 mg/dm^3 were diluted to final concentrations of 5, 10, 20 and 50 mg/dm^3 and the peak areas and the migration times were compared. The solutions were injected twice repeatedly and the values for calibration curve were calculated from mean values of repeated injection. The values of correlation coefficients gave an evidence of the linearity of calibration curves for both daidzein and genistein at concentrations ranging from 5 to 100 mg per dm^3 (Figs. 2 and 3). For the quantitative determination of daidzein and genistein, *p*-nitrophenol with migration time 25.19 min was used as an internal standard. The dif-

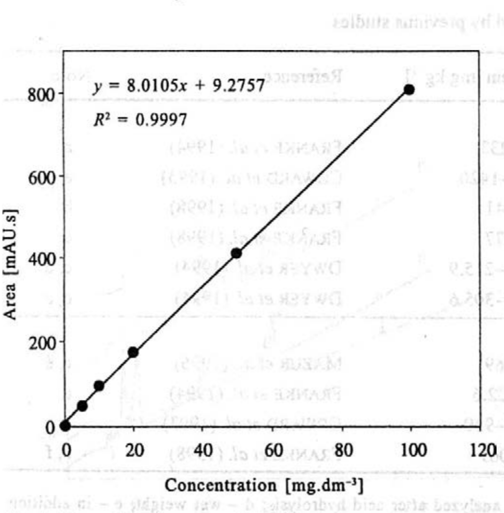


Fig. 2. The calibration curve for daidzein

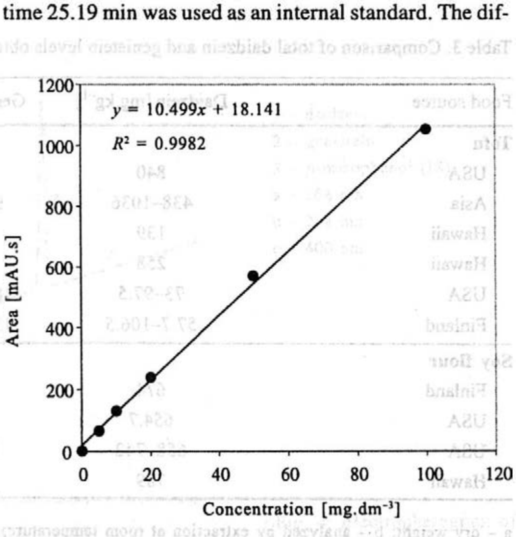


Fig. 3. The calibration curve for genistein

ferences between migration times of isoflavones and that of *p*-nitrophenol resulted in a "separation window" in the electrophoregram, where other compounds could be detected (Fig. 1). In the case of complex samples, the interference of unknown components should be anticipated. The following relative response factors were estimated: daidzein 0.519; genistein 0.755; *p*-nitrophenol 1.000. The limit of detection was calculated as S/N = 3 and limit of quantification as S/N = 10, respectively (Table 1). The minimum detectable limits (Table 1) found for daidzein and genistein are comparable to that of genistein (0.4 mg/dm^3) reported in literature (SHIHABI *et al.* 1994). The theoretical plate number of both analytes in the HPCE analyses was ca. 90 000, showing slightly better performance to the published one (MORITA, SAWADA 1993).

Table 1. The performance and noise determination for HPCE analysis of a standard mixture of daidzein and genistein[a]

	Daidzein	Genistein	*p*-nitro-phenol
Noise (6 × SD)[b] (mAU)	0.1646	0.1648	0.2122
Drift (mAU/h)	3.394	2.947	1.519
Migration time (min)	13.52	13.87	20.19
Theoretical plate number	93 672	88 241	73 011
Resolution	1.72	1.61	–
S/N	47.20	57.40	23.1
Limit of detection (mg/dm^3)	0.32	0.26	–
Limit of quantification (mg/dm^3)	1.00	0.90	–

[a] concentration of both isoflavones was 5 mg/dm^3
[b] time range from 15 to 20 min

As the concentration of genistein and daidzein in their natural source, plants, is sufficiently high, the food material could be analyzed by HPCE method using only simple extraction. Employing the developed HPCE method, genistein and daidzein were determined in food rich in isoflavones. The samples of tofu and soy flour were extracted by diluted acetonitrile (MURPHY 1981) and hydrolyzed enzymatically (Table 2) because enzymatic hydrolysis was found more efficient than the acid hydrolysis by trifluoroacetic acid. The acid hydrolysis was performed under elevated temperature and isoflavones were probably partially destroyed. The daidzein and genistein

Table 2. Mean total concentration of isoflavones in soy food

	Genistein [mg.kg^{-1} DW]			Daidzein [mg.kg^{-1} DW]		
	average	SD	RSD [%]	average	SD	RSD [%]
Soy flour[a]	53.0	4.9	9.33	68.0	8.0	11.76
Soy flour[b]	406.1	58.9	14.51	516.1	80.9	15.68
Tofu[a]	76.4	2.3	3.03	74.0	1.6	2.14
Tofu[b]	726.9	38.4	5.28	644.5	2.4	0.37

[a] without hydrolysis; [b] with enzymatic hydrolysis

Table 3. Comparison of total daidzein and genistein levels obtained by previous studies

Food source	Daidzein [mg.kg^{-1}]	Genistein [mg.kg^{-1}]	Reference	Note
Tofu				
USA	840	1233	FRANKE *et al.* (1994)	a
Asia	438–1036	910–1420	COWARD *et al.* (1993)	a
Hawaii	139	141	FRANKE *et al.* (1998)	b
Hawaii	258	377	FRANKE *et al.* (1998)	c
USA	73–97.5	187.4–215.9	DWYER *et al.* (1994)	c, d
Finland	57.7–106.5	158.6–305.6	DWYER *et al.* (1994)	c, d
Soy flour				
Finland	674	969	MAZUR *et al.* (1996)	c, e
USA	654.7	1122.6	FRANKE *et al.* (1994)	a
USA	658–742	837–939	COWARD *et al.* (1993)	a
Hawaii	789	1069	FRANKE *et al.* (1998)	b, f

a – dry weight; b – analyzed by extraction at room temperature; c – analyzed after acid hydrolysis; d – wet weight; e – in addition, 0.81 mg/kg of biochanin A and 0.09 mg/kg of coumestrol; f – in addition, 213 mg/kg of glycitein peaks were verified by spiking the sample with pure standards, because the repeatability of migration times was rather poor. The identification and the peak purity were checked by comparison of absorbance patterns obtained by diode array detection with UV spectra of authentical samples. The total isoflavone levels approximately correspond with the data published (Table 3), if the high variability due the differences in environmental conditions and genetic dispositions of the food and plants has been taken into account. Moreover, it is known, that the total levels of isoflavones can vary even within the same food item, collected from different markets (RSD = 1–101%, FRANKE *et al.* 1998). These observations suggest that detailed and specific soy food analyses are required for studies measuring dietary isoflavone exposure. The ratio of genistein : daidzein of approximately 1.2 : 1 was found (Table 2) in both hydrolyzed samples which is in good agreement with earlier reported ratio of 1 : 1 (FRANKE *et al.* 1998). It seems that the relative content of isoflavones could be kept rather constant. Precision of the proposed CE method (Table 2) was found to be within accepted limits for phytoestrogens analyses. In comparison, the RSD values between 3% and 11% (FRANKE *et al.* 1994) or between 3% and 16% (MAZUR *et al.* 1996) have been described for the quantitation of phytoestrogens in Legumes by HPLC and GC/MS, respectively.

The reproducibility of HPCE method was further assessed by ten replicate analysis of the tofu extracts and both SD and RSD were calculated (Table 4). The repeatability of extraction was evaluated analogously, the SD and RSD values were calculated for ten replicate extractions; each of extracts was analyzed twice (Table 4). The RSD values were higher than these found for the analyses (Table 2) probably in consequence of different analytical protocol. If the analyses of one kind of food were done in sequence, the results were obviously more precise according to the RSD value. Moreover, the optimized sequence for the analyses of food samples involved alternated injection of two extracts followed by the standard solution in order to minimize the problem caused by matrix effect.

A difficult problem concerned the drift of baseline during the analyses of biological samples and the rather per-

Table 4. The statistic evaluation of precision of HPCE analysis[a]

	x_{min} [mg.kg^{-1}]	x_{max} [mg.kg^{-1}]	x_{av} [mg.kg^{-1}]	SD [mg.kg^{-1}]	RSD [%]
Replicate extraction of tofu					
Genistein	97.1	150.7	121.0	18.6	15.39
Daidzein	78.6	148.7	117.4	26.6	22.67
Replicate injection of tofu extract					
Genistein	77.0	105.6	93.1	11.4	12.26
Daidzein	72.5	91.0	80.0	10.6	13.26

[a]without hydrolysis

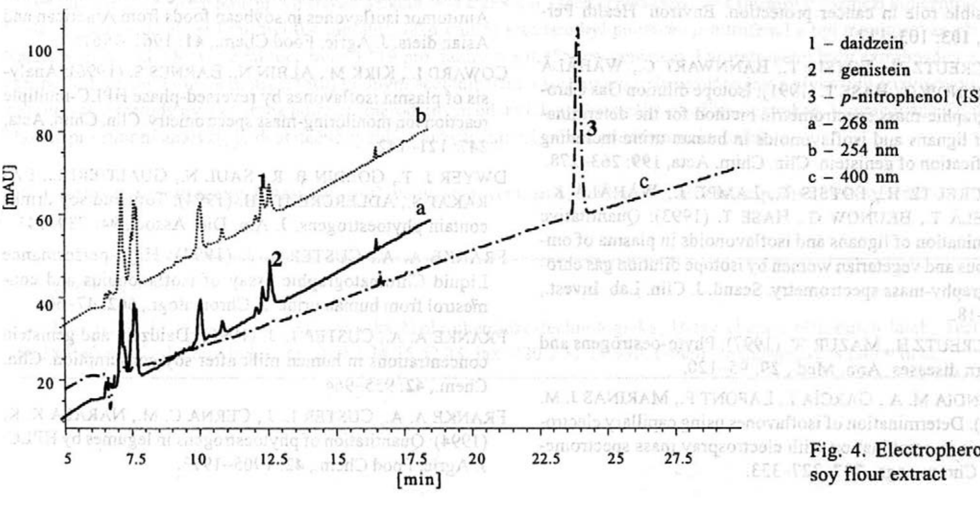


Fig. 4. Electropherogram of soy flour extract

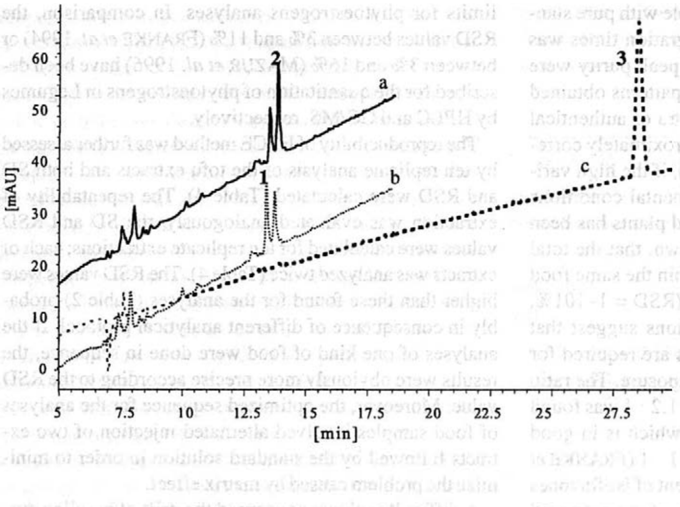


Fig. 5 Electropherogram of tofu extract

1 – daidzein
2 – genistein
3 – *p*-nitrophenol (IS)
a – 268 nm
b – 254 nm
c – 400 nm

manent decreasing of migration times, although the changes in the resolution of analytes were negligible. Evidently, the matrix composition can affect the quality of the inner capillary wall and thus cause the nonstability of baseline (Figs. 4 and 5). Commonly used 0.1M solution of sodium hydroxide was not adequate for good capillary washing in our case. A 1.0M solution of sodium hydroxide was likely to provide more effective washing. However, considerable equilibration time was needed between runs to achieve at least acceptable electroosmotic flow reproducibility.

This HPCE method seems to be reliable enough for routine analyses as the promising approach for analysis of phytoestrogens in food.

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Souhrn

VÄNTTINEN K., MORAVCOVÁ J. (1999): **Fytoestrogeny v sójových potravinách: stanovení daidzeinu a genisteinu kapilární elektroforézou**. Czech J. Food Sci., **17**: 61–67.

Byla vypracována rychlá a selektivní analytická metoda kapilární elektroforézy pro stanovení hlavních fytoestrogenů obsažených v potravinách. Izoflavony daidzein a genistein byly separovány na křemenné kapiláře ve 200mM borátovém pufru a sledovány detektorem diodového pole při vlnových délkách 254 a 268 nm. Odezva detektoru byla lineární v rozmezí koncentrací analytů 5–100 mg/dm^3 a detekční limit byl 0,4 mg/dm^3. Jako vnitřní standard byl používán *p*-nitrofenol a byl monitorován při 400 nm. Nalezené relativní odezvové faktory byly 0,519 pro daidzein a 0,755 pro genistein. Fytoestrogeny byly extrahovány z potravin vyrobených ze sóji (sójová mouka, tofu) vodným acetonitrilem a isoflavony byly uvolňovány z glykosidů enzymovou hydrolýzou. Přesnost metody byla ověřena pomocí směrodatných odchylek opakovaného nástřiku a extrakce. Vypracovaná CE metoda je vhodná pro rutinní analýzy, je dostatečně rychlá a nevyžaduje náročnou předseparaci vzorků.

Klíčová slova: fytoestrogeny; potraviny; izoflavony; stanovení; sója; daidzein; genistein; kapilární elektroforéza

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Determination of the Oxidative Stability of Fats and Oils using the Oxipres Apparatus*

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Abstract

TROJÁKOVÁ L., RÉBLOVÁ Z., POKORNÝ J. (1999): **Determination of the oxidative stability of fats and oils using the Oxipres apparatus.** Czech J. Food Sci., 17: 68–72.

The application of an Oxipres apparatus for the determination of oxidative stability of fats and oils gives results similar to those obtained in frying experiments as the temperature is in the range of 100–150 °C, and the access of oxygen is limited by diffusion. The repeatability was about of the same order as in the case of the Schaal Oven Test in the case of rapeseed and sunflower oils or lard. The optimum initial oxygen pressure is 0.5 MPa, the optimum sample weight 5 g. The temperature of 100 °C is sufficient to finish most experiments within a working day. The relation between graphically determined induction periods obtained using an Oxipres and those of the Schaal Oven Test at 60 °C was semilogarithmic.

Key words: antioxidants; autoxidation; fats; lard; oils, edible; Oxipres apparatus; stability against oxidation

The stability against oxidative deterioration is a very important characteristic of edible fats and oils. A storage experiment would be the best way to determine it, but it would be too long, therefore, accelerated tests are generally used. A traditional method of its determination is the Schaal Oven Test (PARDUN & KROLL 1970), which has the advantage of its similarity to storage conditions, however, it takes long time, such as several weeks at 60 °C or several months at 40 °C. Samples are stored in the dark at constant temperature and at free access of oxygen. The repeatability is rather good (POKORNÝ *et al.* 1985), in spite of its simplicity.

Another traditional method is the Active Oxygen Method (AOM, often called Swift test), when samples are bubbled through with a stream of air at 100 or 110 °C, and effluent gases are examined, e.g. sensorically. The method has been standardized (AOCS 1996). An important improvement was a conductometric indication of acids in gases from the reaction tube (HADORN & ZÜRCHNER 1974; ZÜRCHNER 1974). The method could be then automatized (DEMAN & DEMAN 1984), and its precision determined (OOSTEN *et al.* 1981). The optimum temperature was studied in order to shorten the induction period to one working day; the logarithm of the induction period is a linear function of temperature (REYNHOUT 1991; MARINOVA & YANISHLIEVA 1992).

The apparatus Rancimat is based on the same principle, only it is more sophisticated, so that the results are comparable to those obtained with the AOM procedure (LÄUBLI *et al.* 1988a, b). Oxidation conditions of the method are very different from storage conditions, nevertheless, the method is good for preliminary tests of antioxidant activity (LÄUBLI 1994). The temperature dependence of the induction period was semilogarithmic (HASENHUETTL & WAN 1992), similarly like in case of the AOM. The reproducibility was determined using an interlaboratory test (WOESTENBURG & ZAALBERG 1986). The procedure was applied to refined and virgin olive oils (FREGA *et al.* 1995), bakery fats (KLEINERT 1986) or potato chips (BARRERA-ARELLANO & ESTEVES 1992).

The Oxidative Stability Instrument (OSI) is based on the same principle as the Rancimat method (AKOH 1994; HILL & PERKINS 1995), and both methods give similar results (JEBE *et al.* 1993).

Both the AOM and the Rancimat method give results which are in good agreement with one another (LÄUBLI & BRUTTEL 1986; LÄUBLI *et al.* 1988a, b; GUTIÉRREZ 1989). The Rancimat method was compared with ultravio-

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let irradiation (GORDON *et al.* 1994). Longer induction periods were observed with the Rancimat method compared with various chemical indices (RAUEN-MIGUEL *et al.* 1992).

FRANKEL (1993) questioned the suitability of both AOM and the Rancimat method as measures of the keepability under storage conditions. He considers temperatures of 100–110 °C as too high, preferring the Schaal Oven Test at 40–60 °C.

Another approach to the mesurement of antioxidant activities is to determine the oxygen absorption. The ASTM oxygen bomb method consists in exposing the sample to oxygen at high pressure and temperature, and measuring changes of oxygen pressure at a constant volume. It was applied several decades ago for the study of fats and oils (POHLE *et al.* 1963), and a good correlation was observed between the results and those obtained using the AOM procedure. The method could be used not only for bulk fats and oils, but also for solid foods, such as peanuts (BLANKENSHIP *et al.* 1973).

Several other instruments were used, operating at atmospheric pressure with manostatic measurement of the oxygen pressure (DROZDOWSKI & SZUKALSKA 1987). Similar devices were used in Japan (KATO & MASHIO 1980) and in our laboratory (POKORNÝ *et al.* 1974). The oxygen uptake may be measured using gas chromatography (FARIA 1982), polarography (LINGNERT *et al.* 1979) or an oxygen electrode (HIRAYAMA *et al.* 1986).

The ML Oxipres is based on a similar principle, as samples are stored under high pressure of oxygen at higher temperature, and the decrease of pressure on storage is recorded. We studied the use of the apparatus for the study of bulk fats and oils, and compared the results with those of the Schaal Oven Test.

MATERIAL AND METHODS

Material

Refined zero-erucic winter rapeseed (*Brassica napus* L.) and high-linoleic sunflower (*Helianthus annuus* L.) oils and pork lard (produced by hot water extraction) were commercial products, purchased on the market; their characteristics are summarized in Table 1. They were analyzed 3–6 months after their production.

Table 1. Characteristics of fats used in the experiments

Characteristic	Unit	Oil rapeseed	Oil sunflower	Pork lard
Peroxide value	[meq/kg]	0.77	1.17	0.87
Acid value	[mg/g]	0.59	0.08	1.63
Conjugated dienes	[p. c.]	0.49	0.24	0.18
Polar compounds	[p. c.]	1.27	2.46	1.51
Tocopherols	[mg/kg]	350	440	

Analytical Methods

The acid value, peroxide value, and conjugated dienes were determined according to standard procedures (IUPAC 1987), tocopherols by high-performance liquid chromatography (HPLC) according to the standard procedure (IUPAC 1987). Polar lipids were determined using HPLC (PÁNEK *et al.* 1989), modified in the following way: The apparatus Hewlett-Packard No. 1050 (Produced by Hewlett-Packard, Waldbronn, Germany), provided with an autosampler and an integrator HP 3396 Series II, a stainless steel column, 250 × 4 mm, packed with Separon SGX C18, 5 µm (Tessek, Prague); injected volume: 50 µl; mobile phase: acetone–acetonitrile–methanol, 4 : 2 : 1 (v/v/v); flow rate: 1 ml/min; column temperature: 40 °C; differential refractometer HP 1047A, with automatic cell washing after each analysis.

The Schaal Oven Test (25g samples placed in 100 ml beakers) was carried out at 60 °C, with gravimetric detection (POKORNÝ *et al.* 1985).

In the case of the Oxipres method, the apparatus ML Oxipres (Mikrolab Aarhus A/S, Højbjerg, Denmark) was used, provided with two measuring cells. Fat was weighed to the nearest 0.01 g into a reactor tube, the reactor was connected to a pressure bottle of oxygen, washed with oxygen and then filled with oxygen at the defined initial pressure. The tube was then placed into a thermostat, and pressure changes were recorded. The induction period was calculated as shown in Fig. 1. The optimized conditions were as follows: 5.0g samples; initial pressure: 0.5 MPa; temperature: 100 °C.

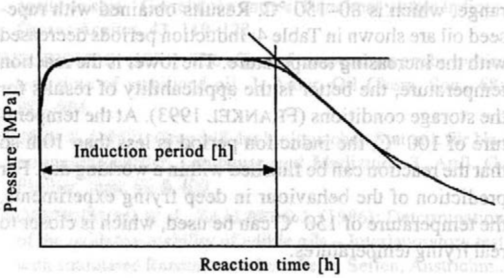

Fig. 1. Oxipres record and way of induction period determination

Statistical Methods

The software Microsoft STATISTICA 6.0 was used; the average difference between duplicates was calculated according to Gini (WEBER 1957).

RESULTS AND DISCUSSION

The effect of the sample weight on the induction period is summarized in Table 2. The values are means of duplicates. Larger sample produces deeper layer of oil, which oxygen has to penetrate by diffusion. No substantial dif-

Table 2. Effect of sample weight [g] on the induction period [h]

Sample weight	Oil rapeseed	Oil sunflower	Pork lard	Average value
3.0	8.10	5.40	2.40	5.30
4.0	8.13	5.38	2.53	5.36
5.0	8.31	5.38	2.54	5.41
6.0	8.07	5.43	2.68	5.39

ferences were observed in the range of 3–6 g so that small variations during weighing have no influence on the result. The induction period obtained with 4–5 g sample gave slightly higher values.

The induction period moderately decreased with increasing initial oxygen pressure (Table 3). The value of 0.5 MPa was chosen as suitable for practical reasons. Small differences in the adjustment of initial pressure (of the order of 0.05 MPa) have no substantial effect on the results.

Table 3. Effect of initial oxygen pressure [MPa] on the induction period [h] of rapeseed oil

Initial pressure	Induction period determination 1	Induction period determination 2
0.30	9.3	9.1
0.40	8.6	8.7
0.50	8.4	8.2
0.55	8.1	8.0

The effect of temperature was studied in the operating range, which is 80–150 °C. Results obtained with rapeseed oil are shown in Table 4. Induction periods decreased with the increasing temperature. The lower is the reaction temperature, the better is the applicability of results for the storage conditions (FRANKEL 1993). At the temperature of 100 °C, the induction period is less than 10 h so that the reaction can be finished within a working day. For prediction of the behaviour in deep frying experiments, the temperature of 150 °C can be used, which is closer to real frying temperatures.

Table 4. Effect of reaction temperature [°C] on the induction period [h]

Temperature	Induction period determination A	Induction period determination B	Average value
80	39.35	39.20	39.28
90	17.55	17.50	17.52
100	8.00	8.50	8.25
110	3.90	2.85	3.38
120	2.00	2.13	2.06
130	1.18	1.16	1.17
140	0.73	0.78	0.76
150	0.57	0.59	0.58

The repeatability of the determination is shown in Table 5. Standard deviations were of the same order; higher relative values in the case of lard are due to shorter induction period as absolute values are nearly the same as in the case of oils. Relative differences between the duplicates are about the same as in the case of the Schaal Oven Test (POKORNÝ *et al.* 1985).

Table 5. Repeatability of induction period measurements

Material	Oil rapeseed	Oil sunflower	Pork lard
Number of determinations	10	8	8
Mean value [h]	8.31	5.38	2.54
Standard deviation [h]	0.143	0.104	0.145
Variation coefficient [p. c.]	1.72	1.93	5.71
Mean difference between duplicates [h]	0.165	0.112	0.157

In a series of rapeseed oil samples, stabilized by different antioxidants, the stabilities against oxidation were determined both by the Schaal Oven Test at 60 °C and by the Oxipres method at 100 °C. The plot of the results (Fig. 2) is not a straight line, but a semilogarithmic relation was obtained, not differing much from the linearity. This course may be explained by the semilogarithmic relation between the induction period and the temperature (MARINOVA & YANISHLIEVA 1992). The course is due to the effect of temperature on the rate of oxygen diffusion like under the experimental conditions, the diffusion is the rate determining factor.

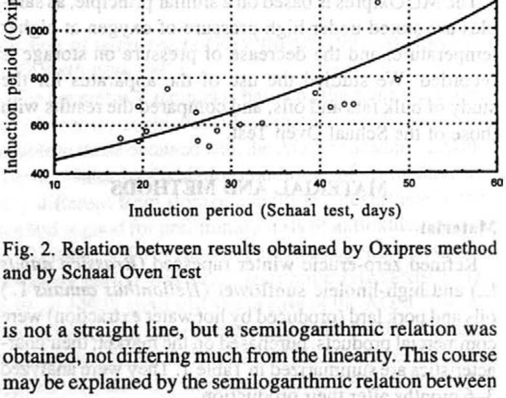

Fig. 2. Relation between results obtained by Oxipres method and by Schaal Oven Test

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Souhrn

TROJÁKOVÁ L., RÉBLOVÁ Z., POKORNÝ J. (1999): **Stanovení oxidační stability tuků a olejů přístrojem Oxipres.** Czech J. Food Sci., 17: 68–72.

Pro stanovení stability tuků a olejů byla vyzkoušena metoda s použitím přístroje ML Oxipres (Aarhus, DK), jejíž princip spočívá v tom, že je vzorek vystaven bez míchání kyslíku za zvýšeného tlaku a teploty. Jako optimální se doporučuje na základě našich výsledků navážka 5 g tuku, tlak kyslíku 0,5 MPa a teplota 100 °C, pro kontrolu stability za podmínek smažení 150 °C. Za těchto podmínek většinou proběhne stanovení během jednoho pracovního dne. Opakovatelnost stanovení byla zjištěna u řepkového a slunečnicového oleje a u vepřového sádla. Řádově odpovídá přesnosti Schaalovy zkoušky. Mezi výsledky metodou Oxipres při 100 °C a Schaalovou zkouškou při 60 °C existuje semilogaritmická závislost, která se jen málo liší od lineární.

Klíčová slova: antioxidanty; autooxidace; oleje jedlé; Oxipres; sádlo vepřové; Schaalova zkouška; stabilita; tuky

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The Determination of Zinc Content in Wines by Differential Pulse Anodic Stripping Voltammetry (DPASV)

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Abstract

DOSTÁLEK P., KOPLÍK R., PATZAK M. (1999): **The determination of zinc content in wines by differential pulse anodic stripping voltammetry (DPASV).** Czech J. Food Sci., **17**: 73–76.

The zinc content of different types wines from Znojmo region was determined by differential pulse anodic stripping voltammetry without sample pre-treatment. 45 different wines were analysed. Almost all samples of wine contained less than 1.5 mg/l of zinc (from 0.05 to 3.71 mg/l). The mean zinc concentrations were 1.01 and 1.17 mg/l for white and red wines, respectively. The average of zinc level in sparkling and fortified wines were 1.44 and 1.21 mg/l respectively.

Key words: **zinc; wine; differential pulse anodic stripping voltammetry**

The inorganic compound content in wine usually does not exceed 5 g/l (LINSKENS *et al.* 1988). The typical concentration of inorganic elements is shown in Table 1.

There are various methods to determine zinc concentration in organic materials. In the majority of cases these methods are based on the decomposition of samples. These analyses are considerably time consuming.

One of the most useful methods for determination of trace elements is flame or flameless atomic absorption spectrometry (AAS). These methods usually require sample pre-treatment. Direct determination of copper in wine is difficult due to the presence of sugar and alcohol (MÉRANGER *et al.* 1968). Voltammetric methods, namely differential pulse polarography (DPP) and voltammetry, enable very sensitive determination of some metals, e.g. Pb and Cu (NÜRNBERG 1982). Unfortunatelly, the application of these methods to analysis of foodstuffs and beverages usually requires complete sample decomposition to remove the organic matrix (ADELOJU *et al.* 1985).

DPP was used for simultaneous determination of Cu, Pb and Zn in wine at 0.03–0.08 mg/l levels. Samples were evaporated and decomposed in a HNO_3/H_2O_2 mixture. The polarographic measurement was then performed in potassium hydrogen phthalate solution at pH value 3.1 (BRUNO *et al.* 1978). Higher sensitivities were achieved using stripping voltammetry, so that smaller sample volumes (1–10 ml) could be used (MANNINO 1982; OEHME & LUND 1979; POPKO *et al.* 1978). These elements can also be determined in wine by differential pulse anodic stripping voltammetry (DPASV) after UV-irradiation of the acidified sample to which H_2O_2 has been added (GOLIMOWSKI *et al.* 1979). Direct determination of copper and lead in wines was also published (DOSTÁLEK *et al.* 1994, 1997).

This paper is concerned with the monitoring of zinc content in wines from Znojmo region in the Czech Republic. Among the white sorts mainly Rhine Riesling, Traminer, Müller Thurgau and Chardonnay are cultivated here; among the blue sorts are Vavřinecké, Portugieser and Frankovka (FARKAŠ 1988).

45 samples of wines from Znojmo region were analysed for zinc content. Direct measurement by differential pulse anodic stripping voltammetry without sample pre-treatment was applied.

MATERIALS AND METHODS

Chemicals and Glassware

Standard zinc solution (c = 1 g/l in 0.3 mol/l HNO_3) for atomic absorption spectrometry was used for preparation of working standards. Hydrochloric acid (36%), formic acid and acetic acid with a maximum zinc content 1 µg/l were used for preparation of supporting electrolyte. Except for nitric acid analytical grade, used for soaking of glassware, all other chemicals were products of the company Analytika, Praha, of the highest purity. All glassware were decontaminated before use by three weeks soaking in dilute HNO_3 (1 : 10) and thorough rinsing with redistilled water. All aqueous solutions were prepared using redistilled water.

Tab. 1. The usual wine inorganic elemnt content [mg/l] (ESCHNAUER *et al.* 1989)

Macro elements	Trace elements				Ultra trace elements
10–1000	1–10	0.1–1	0.01–0.1	0.001–0.01	< 0.001
K 370–1120	B 2–5.0	Al 0.50–0.9	As 0.003–0.02	Co 0.001–0.02	Sb 0.00600
Mg 60–140	Fe 1.0	F 0.05–0.5	Ba 0.04–0.30	Mo 0.001–0.01	Be 0.00008
Ca 70–140	Cu 0.5	I 0.10–0.6	Pb 0.03–0.10	Ag 0.005–0.02	Cd 0.00100
Na 7–15	Mn 1.5–5.0	Rb 0.20–4.2	Br 0.01–0.70		Cs 0.00270
C 100–120	Si 1.5–6.0	Sr 0.20–3.5	Cr 0.03–0.06		Au 0.00006
P 130–230	Zn 0.5–3.5	Ti 0.04–0.3	Li 0.01–0.20		Hf 0.00070
S 5–10			Ni 0.03–0.05		Nb 0.00100
Cl 20–80			V 0.06–0.26		Hg 0.00005
			Sn 0.01–0.70		Se 0.00060
					W 0.00300
					Bi 0.00015

Sampling

In 1994 and 1995, 45 bottles of different wines originating from Znojmo region were analysed. Each sample of wine was described by its origin, sort and vintage. The samples included wines in bottles with the same type of cork closure.

Procedure of Zinc Determination in Wine by DPASV

The amount of 5 ml of wine was transferred to a polarographic cell. After oxygen removal from the mixture by 5 min bubbling with nitrogen, zinc was determined by DPASV using conditions described below. The quantitation was made by the standard addition technique.

Measuring Instrument and Conditions

A polarographic analyzer PA-3 equipped with static mercury drop electrode – SMDE1 (Laboratorní přístroje Praha) was used for voltammetric determination. Electrochemical data station POLAR 2.5 was used for data evaluation. Parameters of determination were as follows:

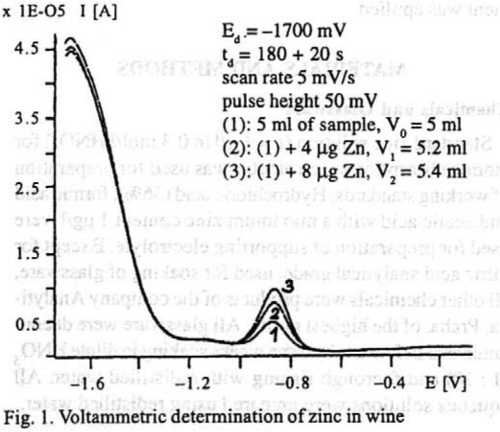

Fig. 1. Voltammetric determination of zinc in wine

- working electrode: HMDE
- reference electrode: Ag/AgCl/sat. KCl
- mode DPASV
- drop expansion time 0.1 s
- pulse duration 100 ms
- pulse height 50 ms
- current sampling time 20 ms
- deposition potential –1700 mV
- final potential 0 mV
- deposition time 60–180 s
- equilibration time 20 s
- current range 500 nA
- scan rate 5 or 20 mV/s

Zinc Determination in Wine by AAS

Determinations of Zn by AAS were performed in Central AAS Laboratory in the Institute of Chemical Technology with atomic absorption spectrometer Varian Spectr AA300. Zinc content was determined by flame AAS with the following parameters: flame – acetylene-air, wavelength 213.9 nm, slit width 0.2 nm.

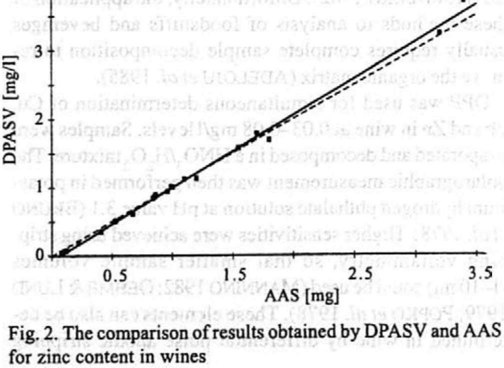

Fig. 2. The comparison of results obtained by DPASV and AAS for zinc content in wines

RESULTS AND DISCUSSION

Stripping voltammetry is often used for zinc measurement in food samples decomposed by wet or dry method (ADELOJU 1989). This highly sensitive technique was chosen for zinc determination in wine samples without any pre-treatment.

In the first part of experiments the addition of acetic acid and formic acid to wine samples (to achieve 0.5 mol/l acid concentration) was tested. Zinc was deposited from these solutions by electrolysis at –1400 mV and determined by anodic stripping voltammetry. Under these conditions the stripping peaks of zinc were quite symmetrical. The analogous peak shapes were obtained when wines were analysed without addition of an acid, but analytical results were slightly lower in this case (decrease approximately 10%).

Wine samples were analysed for zinc content by AAS method too. The comparison of AAS and DPASV (deposition potential –1400 mV) methods showed that voltammetric analysis provides lower results. This is probably caused by the fact that a part of zinc content in wine is not available for electrochemical determination because stable zinc complexes occur in wine. Zinc bound in these stable complexes is not deposited on working electrode in case when electrolysis is carried out at –1400 mV. Sample acidification by acetic acid or formic acid has only a slight effect on the breakdown of stable zinc compounds. Acidification of wine by hydrochloric acid cannot be used because hydrogen is liberated on mercury electrode and therefore zinc peak is overlapped.

In order to investigate the influence of measurement parameters on the result of voltammetric analysis the effect of deposition potential on zinc peak height was ascertained. It was found that results of voltammetric analysis were in good agreement with those obtained by AAS when potential of electrolysis is equal to or more negative than –1650 mV. Therefore the value –1700 mV was selected in final analytical procedure. For most samples 1 minute electrolytic deposition is appropriate. An example of zinc determination in wine is given in Fig. 1.

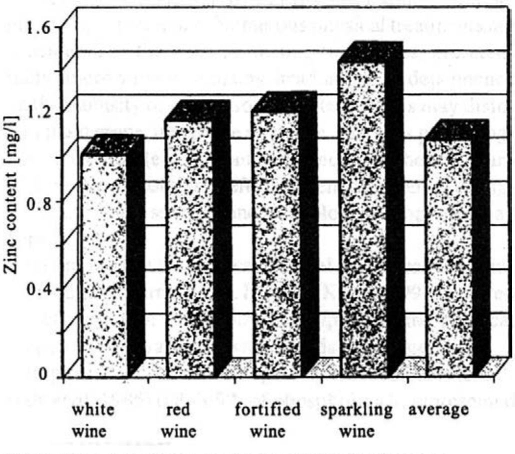

Fig. 3. The average zinc contents in several wine types

A total of 45 bottles of 45 different wines were analysed. Each sample of wine was analysed minimally of five times. The results of measurements on white wine, red wine, sparkling wines and fortified wines are presented in Fig. 3. Accuracy of the voltammetric method was verified by comparison with an independent AAS method. The relation between results of DPASV and AAS analyses is shown in Fig. 2 and can be represented by the formula: $C_{Zn}(DPASV) = (-0.03 \pm 0.05) + (1.04 \pm 0.07)\ C_{Zn}(AAS)$.

It is evident that both methods lead to almost identical results.

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Souhrn

DOSTÁLEK P., KOPLÍK R., PATZAK M. (1999): **Stanovení obsahu zinku ve vínech diferenční pulzní anodickou rozpouštěcí voltametrií (DPASV).** Czech J. Food Sci., 17: 73–76.

Obsah zinku ve vínech pocházejících ze znojemské oblasti byl stanoven diferenční pulzní anodickou rozpouštěcí voltametrií bez předchozí úpravy vzorků. Téměř všechny vzorky vín obsahovaly méně než 1,5 mg zinku v 1 litru (od 0,05 do 3,71 mg/l). Průměrná koncentrace zinku v bílých a červených vínech jsou 1,01 a 1,17 mg/l. Průměrný obsah v šumivých vínech byl 1,44 mg/l a v dezertních vínech 1,21 mg/l.

Klíčová slova: zinek; víno; diferenční pulzní anodická rozpouštěcí voltametrie

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Effects of Microwave and γ-Irradiation Treatment on the Rape Seed*

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Abstract

NOVOTNÁ Z., VALENTOVÁ O., SVOBODA Z., SCHWARZ W., KÁŠ J. (1999): **Effects of microwave and γ-irradiation treatment on the rape seed.** Czech J. Food Sci., 17: 77–80.

In order to protect rape seed against unfavourable quality changes during storage and processing, microwave and γ-radiation treatment was applied. The effect of this treatment was evaluated by investigating certain biochemical changes in the rape extracts. Whole seeds with moisture content of 8% were treated by microwave heating under controlled conditions. Approximately 10 min of treatment was required to decrease phospholipase D (PLD) activity to 10% of its original value and the protein profile of the rape extracts was changed as well. Neither PLD activity nor the protein pattern of the extracts was changed by γ-radiation.

Key words: rape seed; phospholipase D; microwave treatment; γ-irradiation

Oilseeds are ancient crops having many uses as food and fodder and resources for the diverse industrial applications covering production of plastics, fuels, detergents, lubricants and cosmetics. Rape seed represents one of the most important oil seeds in EC and Central Europe where almost 1/3 of the total world crop is harvested (30 millions of tons in 1991). The world production increased by more than one third at the last decade (MURPHY 1993). On a world wide production basis rape seed ranks fifth among vegetable oils (after soybean seeds, sunflower seeds, peanuts). In the Czech Republic, rape seed is surely the oil seed number one (production of about 1/2 millions of tons per year).

Keeping the good quality of the seeds requires suitable pre-storage treatments. Numerous physical treatments are considered as the most promising techniques, preferentially microwave or ionising irradiation. In dependence on the intensity of irradiation these techniques may disinfect plant crops, inactivate enzymes, suppress physiological activities (e.g. sprouting), decrease the moisture content, etc. In contrast, defects in genetic material, changes in nutritional, sensoric and technological properties may appear.

It is known that the physicochemical properties of proteins are modified by irradiation. BYUN & KANG (1994) reported the efficiency of irradiation for improving the physical properties of soya beans with regards to its processing.

Rape oil contains according to the recent studies (HOUGEN *et al.* 1985) 0.8–3.5% of phospholipids, represented mainly by phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. All mentioned phospholipids are potential substrates for phospholipase D, a lipolytic enzyme hydrolysing the phosphorus ester linkage between 1,2 diacylglycero-*sn*-3 phosphate and the hydrophilic alcohol substituent. The product of this enzyme reaction is phosphatidic acid (nonhydratable phospholipid, NHP) which affects industrial oil processing and flavour of final product in an undesirable way. NHP in crude oil are not readily removed during the typical commercial degumming process and require special and more expensive processing steps to facilitate their removal. LIST *et al.* (1990) indicated that inactivation of phospholipase D (PLD) provided either by live steam or by microwave heating (prior to extraction with hexane yields crude oils with extremely low levels of NHP). Thus it was proved that heat, moisture and enzyme activity are important factors contributing to the formation of nonhydratable phospholipids in extracted crude oils. Our recent investigations (NOVOTNÁ *et al.* 1996) has shown that phospholipase D within the oil processing keeps its activity and therefore also the ability to produce phosphatidic acid at least up to the third or fourth step of the industrial process. It means that the amount of phosphatidic acid within the oil production may increase until the enzyme is not inactivated.

Some observations concerning the effects of microwave treatments and γ-irradiation on the activity of phospholipase D and protein profiles in the rape seed are reported here.

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MATERIAL AND METHODS

Plant Material

Seeds of winter rape plant (*Brassica napus*, cv. Lirajet), harvested in 1998 and dried to 8% of water content, were used in all experiments. Samples of seeds were obtained from the Czech Agriculture University in Prague.

Assay of PLD Activity

Substrate emulsion: 40 mg of L-α-phosphatidylcholine, type X-E from dried egg yolk (Sigma) were dissolved in 0.5 ml of ethanol and the solvent was evaporated under nitrogen atmosphere. Then 0.33 ml of 1.83M $CaCl_2$ solution and 0.67 ml of 0.4M Tris-acetate buffer pH 6.0 containing 13mM SDS (sodium dodecyl sulfate), were added. The mixture was emulsified on vortex for 2 minutes.

Incubation: The assay mixture contained 0.1 ml of 0.2M MES (2-N-Morpholinoethanesulfonic acid) buffer pH 6.0, 0.1 ml of substrate emulsion, 0.1 ml of TritonX-100, 0.02 ml of 0.173M SDS and 0.2 ml of the extract. The assay mixture was incubated at 37 °C for 10 min and the reaction was terminated by adding 0.2 ml of 50mM EDTA (ethylenediaminetetraacetic acid) in 1M Tris-HCl buffer pH 8.0 and subsequent boiling for 5 minutes.

Determination of choline: The amount of choline released by PLD action was determined using a choline biosensor (VRBOVÁ *et al.* 1993). The biosensor was inserted into a double coated reaction vessel. Incubation mixture (0.1 ml) was added to the stirred reaction vessel (30 °C) containing 1.4 ml of 0.1M Tris-HCl buffer pH 8.0. The concentration of choline was calculated from a calibration curve established for the standard of choline chloride solutions.

Preparation of Extracts

Phospholipase D was isolated from rape seed by a modification of the method described by KOUZEH KANANI *et al.* (1985). 5 g of the rape seed samples were grownd three times for 20 s (in a coffee grinder), suspended in 25 ml of 0.05M Tris-HCl buffer pH 7.4 containing 1 mM EDTA and 1 mM DTT (dithiothreitol) and shaked at 4 °C for 45 minutes. The mixture was filtered through cheese cloth and centrifuged at 17 600 g at 4 °C for 30 minutes. The floating fat cake was removed and the supernatant was used for the activity measurements.

Microwave Treatment

Microwave treatment was done in collaboration with the Food Research Institute Prague. Samples of the whole seeds were treated by microwave heating using Moulinex Micro/Chef FM2915 Q (850 W, 2450 MHz). The samples were placed to 50 ml beaker (diameter 40 mm). Depth of the seed layer was 12 mm and the treatment was performed in the intervals within 1 to 10 min. Temperature changes of samples were measured during the treatment with an optic fibre system Luxtron 755. Two optical sondes were placed in the centre of the beaker at the depth of 3 mm and 8 mm.

γ-Irradiation of Seeds

This treatment was done in collaboration with the Institute of Nuclear Research (Řež near Prague). Samples of rape seeds (50 g) were treated with two doses rates (0.13 and 0.93 kGy/h) by ^{60}Co γ-irradiator. The dose levels applied were 0.5, 1 and 5 kGy.

Determination of Proteins

Protein content in the rape seed extracts was determined by the Bradford method (BRADFORD 1975) in the format adapted for microtitre plate reader.

Electrophoresis (SDS PAGE)

Electrophoresis was performed in 10 % SDS-polyacrylamide minigels (5% stacking, 0.75 mm thick) with a constant voltage of 180 V. The extracts were diluted with distilled water to the concentration 1 mg of protein per ml and SDS gel loading buffer was added (1/1, v/v). Samples were heated for 3 min to 100 °C. After electrophoresis the proteins were stained with Coomassie blue G-250 according to BLAKESLEY & BOEZI (1987).

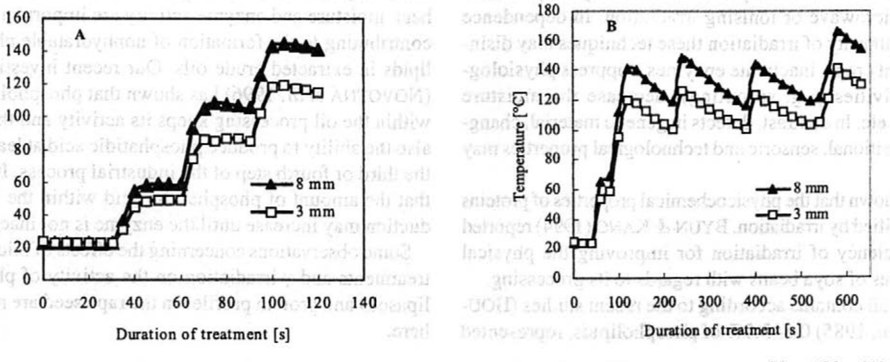

Fig. 1. Temperature changes in rape seeds during microwave treatment. Duration of the treatment was either 120 s (A) or 630 s (B). Optic fibre sensor was placed in the depth of 3 mm or 8 mm, respectively

RESULTS AND DISCUSSION

Changes of Temperature during Microwave Treatment

Time dependence of temperature changes during the microwave treatment of seeds is demonstrated in Fig. 1A, B. The treatment was performed in all cases under the regime for defrosting, it means that the magnetron was on/off in 5 s or 25 s intervals respectively. The temperature of about 100 °C was reached under these conditions within 120 s. The increase of temperature in the samples was higher close to the bottom of the beaker.

The Effect of Microwave Treatment on PLD and Soluble Protein Pattern in Rape Seed Samples

The samples of seeds and flakes (obtained from Setuza during oil production) treated with microwave irradiation as described in detail in the previous chapter were investigated for the remaining phospholipase D activity. Despite the high temperature achieved within two minutes (120–140 °C), the activity in seeds decreased only to 35% of the original value. When the seeds were exposed to this high temperature for a longer period (up to 10 min), the activity dropped to approximately 10% (Fig. 2). This can probably be explained by relatively very high stability of this enzyme in its natural "environment". This is in good agreement with our previous findings (NOVOTNÁ *et al.* 1996) that rape seed phospholipase D possesses very high stability during the rape seed oil processing.

The protein profiles of the extracts prepared from the microwave treated seeds were evaluated by SDS PAGE (Fig. 3). The electrophoretic patterns of the extracts from irradiated seeds slightly differ when compared to the extract of non-treated seeds. Especially, the bands appearing in the range with lower molecular masses (20–35 kDa) are less intensive or disappearing. It is not known, however, that the changes in enzyme activity and protein profile may affect oil processing and the quality of the product.

The Effect of γ-Radiation on Phospholipase D Activity and Soluble Rape Seed Proteins

Changes in phospholipase D activity were studied in the rape seeds treated with the different doses of γ-radiation, the highest dose applied was 5 kGy due to the legislative limits for the food safety (Czech Republic legislation, Low for food and tobbaco products, code no. 110/1997). The final doses of irradiation were achieved by two different dose rates. Any significant changes in the enzyme activity were observed between non-irradiated and irradiated rape seeds (Fig. 4), including the highest intensity of irradiation used (0.93 kGy/h, final dose 5 kGy).

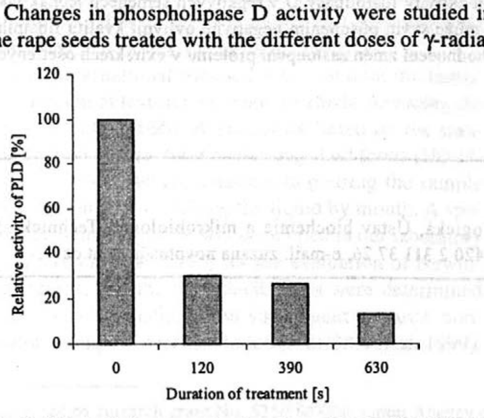

Fig. 2. Effect of microwave treatment on phospholipase D activity in rape seed extracts

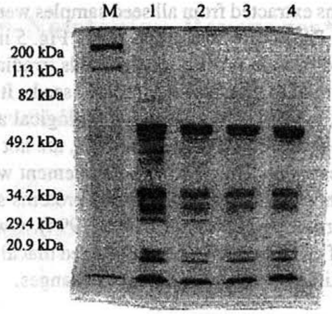

Fig. 3. SDS PAAG electrophoresis of the rape seed extracts treated by microwave heating: lane 1 – non-treated seeds; lane 2, 3, 4 – duration of treatment 120, 390, 630 s, respectively; M – molecular mass standards

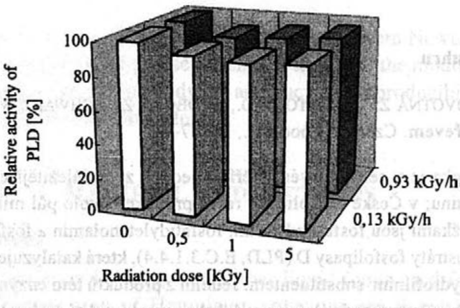

Fig. 4. Effect of γ-radiation treatment on phospholipase D activity in rape seed extracts

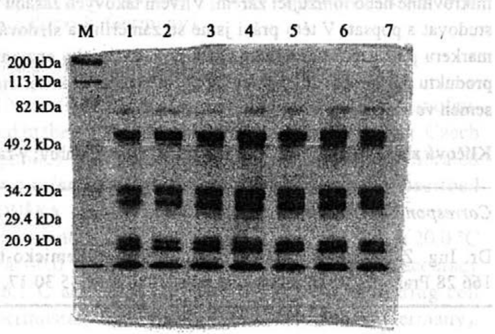

Fig. 5. SDS PAAG electrophoresis of the rape seed extracts treated by γ-radiation: lane 1 – non-treated seeds; lanes 2, 4, 6 – extracts of seeds treated with lower dose 0.13 kGy/h, final doses 0.5, 1, 5 kGy, respectively; lanes 3, 5, 7 – extracts of seeds treated with higher dose 0.93 kGy/h, final doses 0.5, 1, 5 kGy, respectively; M – molecular mass standards

The proteins extracted from all seed samples were analysed by SDS PAGE. Results presented in Fig. 5 indicate that rape seed protein profile for the seeds irradiated up to5 kGy is similar to that from non-treated seeds. It means that proteins, as well as those having biological activity (i.e. phospholipase D as a model enzyme), are not affected by this treatment. This is in good agreement with the results obtained for irradiated soybean proteins studied by CD spectroscopy (BYUN & KANG 1994). Owing to the lability of proteins it may be anticipated that also other food constituents will be resistant to changes.

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We appreciate the help of Dr. J. HOUŠOVÁ from the Food Research Institute Prague and Mr. PEJŠA (Institute of Nuclear Research, Řež near Prague) with the treatment of the samples.

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Souhrn

NOVOTNÁ Z, VALENTOVÁ O., SVOBODA Z., SCHWARZ W., KÁŠ J. (1999): **Ošetření semen řepky g-radiací a mikrovlnným ohřevem**. Czech J. Food Sci., **17**: 77–80.

Řepka je v celosvětovém měřítku jednou z nejdůležitějších olejnin. Produkce řepky se v posledním desetiletí zvýšila o jednu třetinu; v České republice je roční produkce okolo půl mil. tun. Řepkový olej obsahuje asi 0,8–3,5 % lecitinu, jehož hlavními složkami jsou fosfatidylcholin, fosfatidyletanolamin a fosfatidylinositol. Všechny tři jmenované fosfolipidy jsou potenciálními substráty fosfolipasy D (PLD, E.C.3.1.4.4), která katalyzuje hydrolýzu fosfoesterové vazby mezi 1,2 diacylglycero-*sn*-3 fosfátem a hydrofilním substituentem. Jedním z produktů této enzymové reakce je kyselina fosfatidová, jejíž přítomnost ovlivňuje senzorické vlastnosti finálních produktů a působí určité technologické problémy při zpracování olejů. Jednou z možností jak zvýšit kvalitu potravinářských surovin a produktů je jejich ošetření různými fyzikálními technikami. Nejrozšířenějším typem ošetření je mikrovlnné nebo ionizující záření. Vlivem takových zásahu však může dojít v biologickém materiálu k řadě změn, které je nutné studovat a popsat. V této práci jsme se zaměřili na sledování změn aktivity fosfolipasy D v řepkových semenech jednak jako markeru poškození biologické aktivity, jednak jako enzymu, jenž může svým působením negativně ovlivnit kvalitu finálního produktu při zpracování semen. Druhým zvoleným kritériem bylo hodnocení změn zastoupení proteinu v extraktech ošetřených semen ve srovnání s intaktním materiálem.

Klíčová slova: řepka; fosfolipasa D; mikrovlnný ohřev; γ-radiace

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