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Fluorescence screening of antioxidant capacity in pumpkin seed oils and other natural oils

Plant oils provide a rich source of dietary polyunsaturated fatty acids (PUFAs) and mostly lipophilic antioxidants. PUFAs are both in their free form and as components of glycerolipids preferred targets of free radical-induced oxidation, leading to the formation of highly atherogenic compounds. Thus, stabilization of polyunsaturated lipids by radical scavengers in the oils is important in order to avoid pathophysiological side effects of these essential components of our diet. To determine lipid oxidizability and its inhibition by endogenous antioxidants, we developed a simple fluorescence technique. It is based on solubilisation of the oils in aqueous buffer, labeling of the resulting emulsions with a suitable reporter fluorophore, which reflects lipid oxidation, and continuous monitoring of the decomposition process. Using this method, we found that oxidizability of the oils depended only to a limited extent on the content of lipophilic antioxidants. In addition, a smaller fraction of polar (phenolic) compounds showed comparable protective effects, especially in pumpkin seed oil, which is a non-refined product therefore containing antioxidative components that are removed from most other edible oils during processing. Therefore, the contribution of these "minor" compounds has to be taken into account when potential biological effects of plant oils are evaluated.

Keywords: Diphenylhexatriene-phosphatidylcholine, lipid oxidation, phenolics, tocopherols.

Research Paper

1 Introduction

The main components of natural oils are triacylglycerols containing fatty acids of various degrees of unsaturation. Oxidizability of monounsaturated fatty acids by free radicals under biological conditions is not very high, whereas polyunsaturated fatty acids (PUFAs) are highly susceptible in this respect. Lipid oxidation has been recognized as the major source of deteriorative changes in the chemical, sensory and nutritional properties of natural oils. In addition, evidence has been accumulated indicating that lipid oxidation products are very likely to play an important role in the pathogenesis of many diseases and especially atherosclerosis [1, 2]. Lipid oxidation is inhibited *in vitro* and *in vivo* by antioxidants including lipophilic and hydrophilic compounds [3]. Many natural oils contain tocopherols as lipophilic antioxidants as well as minor amounts of more polar substances that are mostly phenolics [4-6]. Although these secondary plant components represent only a minor fraction of the oil, they may exert high biological activity far beyond their antioxidant properties, *e.g.* anticarcinogenic and anti-atherogenic activities [1, 7, 8].

Several methods have previously been developed to study antioxidant capacity of food products. These include measurement of oxygen consumption, formation of lipid peroxides, conjugated dienes, and thiobarbituric acid-reactive substances (TBARS) [5, 9, 10]. Here we report on a novel fluorescence-based method for the determination of antioxidant capacities of natural oils, which directly measures the influence of endogenous and exogenous antioxidants on free radical-induced oxidation of natural oils. This method is simple and suitable for high throughput screening.

The novel technique was applied to evaluate the antioxidant capacity of pumpkin seed oil in comparison with other natural oils. Pumpkin seed oil is a product, which, in contrast to other edible oils, is not refined. Therefore, many (antioxidative) components are still preserved that are removed during processing of most other oils. Pumpkin seed oil is a very popular food ingredient in middle and southwest Europe and is supposed to be beneficial for consumer health. However, solid evidence supporting this assumption is still scarce. We found that pumpkin seed oil exhibits by far the highest antioxidant capacity of all edible oils in our study. The highest contribution to this effect comes from the polar fraction of the oil, which was by far more antioxidative than the classic antioxidant lipids, *e.g.* tocopherols.

2 Materials and methods

Pumpkin seed oils were obtained according to standard procedures from the pumpkin *Cucurbita pepo subspecies*

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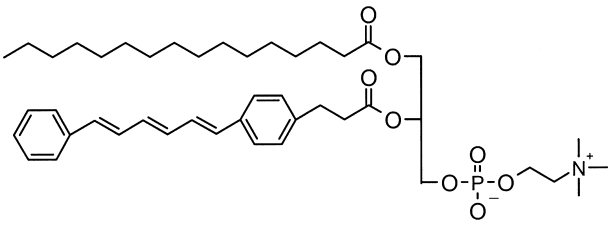

Fig. 1. The fluorescence marker 1-palmitoyl-2-((2-(4-(6-phenyl-*trans*-1,3,5–hexatrienyl)-phenyl)-ethyl)-carbonyl)-*sn*-glycero-3-phosphocholine (DPH-PC)

pepo variatio Styriaca. All oils complied with the requirements of Austrian food law and did not exceed the best before date. Except for the olive oils (from Italy) all oils were of Austrian origin and were pressed in Austria. The sunflower oil rich in oleic acid is a result of the special breeding line Olstaril from *Pioneer* (Parndorf, Austria). The fluorescence marker 1-palmitoyl-2-((2-(4-(6-phenyl-*trans*-1,3,5-hexatrienyl)-phenyl)-ethyl)-carbonyl)-*sn*-glycero-3-phosphocholine (DPH-PC, Fig. 1) was prepared as described previously [11].

2,2'-Azobis-(2-amidinopropane)-hydrochloride (AAPH) was from *Polysciences* (Warrington, USA) and the horseradish peroxidase was from *Sigma-Aldrich* (Vienna, Austria). Tocopherol standards for HPLC were purchased from *VWR International* (Darmstadt, Germany); other tocopherols were purchased from *Sigma-Aldrich* (Vienna, Austria). Methanol (MeOH), *n*-hexane, chloroform ($CHCl_3$) and 1,4-dioxane were purchased from *Promochem* (Wesel, Germany, purity Pico grade, for residue analysis). Argon and Helium 5.0 were obtained from *Air Liquide* (Graz, Austria; purity 5.0) Standard laboratory chemicals were purchased from *Merck* (Darmstadt, Germany) or *Sigma-Aldrich* (Vienna, Austria) and were of analytical grade.

2.1 Extraction of pumpkin seeds

Pumpkin seeds were extracted by Fluidized-Bed-Extraction with a fexIKA 200-control series extractor (*Janke & Kunkel GmbH & Co.KG, IKA-Labortechnik*, Staufen, Germany). The principle of the extraction procedure was described elsewhere [12, 13]. Typically, grinded pumpkin seeds (30±0.5 g) were extracted with 85 ml of *n*-hexane, $CHCl_3$ or a mixture of $CHCl_3$ and MeOH (1/1 v/v). For separation of the extract from the extractive, a fresh polytetrafluoroethylene-filter (47 mm ID, 10-20 µm porosity, fexIKA) was used for each extraction. Six consecutive extraction cycles were performed with all samples, independent of solvent composition. To avoid oxidation during extraction, the collection vessel was flooded with argon prior to extraction and the sample-containing extraction tube was permanently kept under inert gas. The inert gas supply was self-constructed. Some extraction parameters had to be adjusted according to the composition of the extraction solvent (cooling temperature; extraction temperature; extraction temperature hold time). The respective conditions were (40 °C; 80 °C; 10 min) for *n*-hexane, (40 °C; 85 °C; 15 min) for $CHCl_3$ and (35 °C; 80 °C; 15 min) for a mixture of $CHCl_3$ and MeOH (1/1 v/v). At the end of each cooling step, the respective cooling temperature was maintained for three minutes to guarantee complete transfer of the extract into the collection vessel. After extraction, the extracts were transferred into peer-shaped flasks and concentrated using a rotary evaporator (*Büchi*, Fluchs, Swiss). Residual solvent traces were removed under a gentle stream of nitrogen. For the fluorescence oxidation assay and the analytical procedures, the resulting extracts were processed as described for the natural oils.

2.2 Determination of fatty acid composition

The fatty acid pattern of the triacylglycerols was determined *via* transesterification and subsequent analysis of the resulting fatty acid methyl esters (FSME) by gas chromatography. Accordingly, 2 ml 14% BF_3-MeOH and 500 µl benzene were added to 100 µl of the oil (natural oils or pumpkin seed extracts) and the reaction mixture was stirred at 45 °C for 45 min. After addition of 1 ml bidistilled water, the reaction mixture was extracted three times with petroleum ether (40-60 °C bp). From the pooled extracts, 1 µl was injected (split ratio of 1:30) into a gas chromatograph (HP 6890, *Hewlett Packard*, Palo Alto, USA) equipped with an INNOWAX polyethylene glycol capillary column (30 m $\times$ 0.32 mm I.D., 50 µm film thickness; *Hewlett Packard*). The temperature program started isothermal at 160 °C for 5 min, continuing with an increase in temperature of 7.5 °C/min up to 250 °C followed by another isothermal period of 15 min at 250 °C. Helium 5.0 was used as carrier gas (1.4 ml/min) and fractions were detected by flame ionization detector. The injector temperature was 250 °C and the detector temperature was 280 °C. For calibration, a GLC-68B fatty acid ester standard purchased from *Nu-Chek* (Elysian, USA) was used. Data analysis was carried out with Chemstation version 2.0 (*Hewlett Packard*).

2.3 Quantitative analysis of tocopherol

The determination and quantification of tocopherol isomers were accomplished by applying the methodology as described [14, 15]. The analytical equipment consisted of a *Shimadzu* LC-10AT HPLC pump, a *Shimadzu* RF-551 fluorescence detector (*Shimadzu*, Vienna, Austria) and a *Hewlett Packard* 3396 integrator (*Hewlett Packard*, Vienna, Austria). Portions of 5 µl of the diluted sample (about

30 mg/ml *n*-hexane) were injected by means of a Rheodyne 7125 valve and chromatographed on a LiChrosorb® Si 60 column, packed with particles of 5 µm size (*VWR International*). The dimensions of the column were 4.6 mm internal diameter and 15 cm length. The mobile phase was *n*-hexane with an admixture of 2% v/v of 1,4-dioxane. The excitation wavelength was 295 nm and emission was recorded at 335 nm. For quantification purposes a standard stock solution was prepared from a set of 4 individual tocopherol isomers (α-, β-, γ-, and δ-tocopherol) in *n*–hexane at a concentration of about 3.5 mg/ml of each isomer. In order to protect the analytes against oxidation, 25 mg of BHT were added to the standard stock solution. Calibration standards were prepared at nine different concentrations in the range of 0.35 up to 6 ng/µl by appropriate dilution of the stock solution in *n*-hexane.

2.4 Determination of hydrophilic phenolic compounds

Oil samples (natural oils or pumpkin seed extracts) (1.00 g) were extracted using 1.00 ml 80%-MeOH containing 1% hydrochloric acid as a solvent at room temperature under argon atmosphere for 1 h using an orbital shaker set at 200 rpm [16]. The mixture was centrifuged at 1500 g for 3 min, and 800 µl of the supernatant were taken for further analysis. The extraction procedure was repeated three times using 800 µl of fresh extraction solvent each. The resulting extracts were analyzed for phenols by the method of *Folin-Ciocalteu* [17]. Control experiments with exogenously added tocopherols (lipophilic phenols) were carried out to demonstrate that only hydrophilic phenolic compounds are extracted from the oil samples. Therefore, oil samples (pumpkin seed oils and sunflower oils) and pure triacylglycerol mixtures were supplemented with tocopherols (α- and/or γ-) and extracted as described above. The tocopherol content of these samples did not decrease during the extraction procedure (HPLC). On the other hand, the polar extracts of the supplemented samples exhibited the same content of hydrophilic phenolic compounds irrespective of the supplementation of the original oils by tocopherol (analysis see below).

For analysis, 20 µl of the polar extracts were mixed with 150 µl of Folin-Ciocalteu reagent (diluted 10-fold with distilled water). After 5 min incubation at room temperature, 150 µl of sodium bicarbonate (60 g/l) were added and the resultant mixture was left at room temperature for 90 min. Absorbance was measured at 725 nm. For calibration 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (*Trolox*) was used as a standard and results obtained with the oil samples were expressed as *Trolox* equivalents. The total amount of hydrophilic phenolic compounds in the oil was calculated from the results obtained for the individual extracts.

2.5 Fluorescence assay for the determination of antioxidant capacity of oils

Oils were dissolved in $CHCl_3$ (10.0 mg/ml). 270 µl aliquots of these solutions were mixed with 187 µl of Triton X-100 (10.0 mg/ml) and 6.5 µl of a solution of the fluorescence marker DPH-PC (Fig. 1) (0.47 mM) in a 100/1 (v/v) mixture of $CHCl_3$/MeOH. The ratio of oil to label was about 1000:1. At this stage of sample preparation, organic solutions of antioxidants or polar extracts (see above chapter) were added for supplementation experiments. The solvent was removed under a gentle stream of nitrogen. Then 1.00 ml of phosphate buffer (10 mM, pH 6.0, 60 °C) was added to the dried sample followed by short intense mixing under argon atmosphere and consecutive stirring at 60 °C for 10 min and at 37 °C for 2 h.

2.5.1 Fluorescence oxidation assay with AAPH as radical starter

A 100-µl aliquot of the emulsified oil sample was prepared as described above and diluted with 1.35 ml of air-saturated phosphate buffer (10 mM, pH 6.0, 37 °C). The reaction was started by addition of 50 µl of a freshly prepared 90 mM solution of AAPH in bidistilled water. Using a *Shimadzu* RF-5301C fluorimeter (*Shimadzu*, Vienna, Austria) the reaction progress was monitored continuously from the time-dependent decrease in fluorescence at 430 nm (excitation at 354 nm) under permanent stirring at 37 °C.

2.5.2 Fluorescence oxidation assay with horse radish peroxidase (HRP)/H_2O_2 as a prooxidant

A 100-µl aliquot of the emulsified oil sample, which was prepared as described above, was diluted with 1.00 ml of air-saturated phosphate buffer (10 mM, pH 6.0, 37 °C). After addition of 300 µl of a solution of horseradish peroxidase (HRP) (90 µg/ml) in phosphate buffer solution (10 mM, pH 6.0), the reaction was started by addition of 100 µl 0.3% H_2O_2. The decrease in fluorescence intensity was recorded continuously as described above.

The lag times were determined at the point of intersection from the two tangents corresponding to the lag phase (initial phase after addition of prooxidant) and the propagation phase (time-dependent decrease of fluorescence intensity).

3 Results

3.1 Fluorescence assay

A convenient fluorescence assay has been established for the determination of antioxidant capacities of natural oils. For this purpose the oil was co-solubilized with an

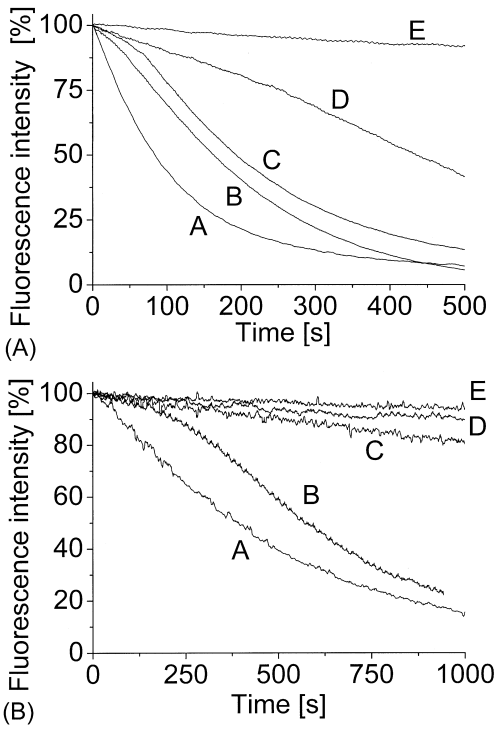

Fig. 2. Typical oxidation assay for edible oils: AAPH (**A**) and HRP/H_2O_2 (**B**) were used as prooxidants. Lipid oxidation was determined from the continuous decrease in fluorescence intensity (see Materials and methods).
(**A**): A: triacylglycerol mixture (triolein/trilinolein, 3/4, w/w), B: olive oil, C: sunflower oil, D: pumpkin seed oil, E: blank without AAPH, sample was pure triacylglycerol.
(**B**): A: triacylglycerol mixture, B: pumpkin seed oil, C: control with H_2O_2 and without HRP, sample was pure triacylglycerol, D: control with H_2O_2 and without HRP, sample was pumpkin seed oil, E: blank with HRP and without H_2O_2.

oxidation-sensitive fluorophore in aqueous buffer using an equimolar amount of Triton X-100 as a detergent. The marker fluorophore was DPH-PC (Fig. 1), as previously described for a lipid oxidation assay in serum [18]. It contains a fluorophore showing the same oxidation susceptibility as compared to natural PUFAs. From the time-dependent decrease of its fluorescence intensity the kinetics of the oxidation process can be determined. Oxidation is preceded by a lag phase, which is representative for the antioxidant capacity of the sample under investigation. It has been measured in this study as a marker for antioxidant capacity of several edible oils. When AAPH was used as a prooxidant, the observed lag times linearly depended on AAPH concentration (data not shown). Alternatively, the enzymatic HRP/H_2O_2 system was used as a prooxidant. Both types of oxidation assay revealed the same major differences between antioxidant capacities of the various natural oils (Fig. 2). The maximum lag times were always found with pumpkin seed oil, irrespective of the prooxidant. Even the pumpkin seed oil showing the lowest lag time still had a significantly higher antioxidant capacity than any other oil in our study ($P < 0.001$, based on students *t*-test) except hemp seed oil (Tab. 1).

3.2 Fatty acid composition of the oils does not affect lag times

Lag times only depend on antioxidant effects and thus should not be affected by the fatty acid composition of pure triacylglycerols, defined triacylglycerol mixtures or natural oils. This assumption was verified for sunflower oils showing a large variation in their content of monounsaturated and polyunsaturated fatty acids. Their lag times solely depended on their antioxidant content. In addition, pure triacylglycerols containing saturated, unsaturated or polyunsaturated fatty acids, but no exogenously added antioxidants, did not show any lag time. In contrast, the propagation rates of oxidation were different. Degradation of the marker fluorophore was faster when the content of (poly)unsaturated lipids in the oil or triacylglycerol was higher.

Model systems containing antioxidant-free triacylglycerol mixtures of triolein and trilinolein (3/4 wt-%), were studied as reference systems since they correspond to the average fatty acid composition of pumpkin seed oils. The respective lipid samples showed immediate oxidation without any lag phase, while the natural oils featured lag times before oxidation started. Again, lag times correlated with the antioxidant content of the lipid mixtures, whereas the propagation rates did not. The oxidation rates of the antioxidant-free triacylglycerol systems (Fig. 2) were higher than the propagation rates of the natural oils.

3.3 Influence of tocopherol content on lag times

Addition of exogenous α-tocopherol and/or γ-tocopherol to pure triacylglycerols resulted in a linear increase of the apparent lag times depending on antioxidant concentration (Fig. 3). On the basis of this calibration plot we calculated the lag times of the natural oils from their tocopherol content. However, a good correlation between the content of endogenous tocopherol (HPLC) with the lag times was only found for pure triacylglycerols (Fig. 3) but not in the case of natural oil samples (Tab. 1). In addition, the measured lag times were always higher than or at least equal to (sunflower oils) the lag times predicted from the total tocopherol content. Therefore, it was concluded that in addition to tocopherols another class of compounds must be responsible for the observed total antioxidant capacities, appearing as specific lag time increments for a given natural oil. This hypothesis was confirmed by supplementation experiments. Increasing amounts of exogenous to-

Tab. 1. Measured lag times, tocopherol contents, fatty acid patterns and *Trolox*-equivalents of different oils and pumpkin seed extracts.

Oil	Lag time[†]	Tocopherol content[‡]				Fatty acid content[‡]			Oil phenolics
		α-	β-	γ-	δ-	saturated	mono-unsaturated	poly-unsaturated	*Trolox* equivalents[#]
	[s]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[%]	[%]	[%]	[µmol/g oil]
Hemp seed oil 1	149 ± 7.3	18	n.d.[§]	847	n.d.	10.6	12.7	76.7	0.76
Hemp seed oil 2	113 ± 4.2	n.d.	n.d.	820	n.d.	10.6	12.9	76.5	0.73
Olive oil 1	8 ± 0.4	86	n.d.	n.d.	n.d.	11.7	78.9	9.4	0.97
Olive oil 2	26 ± 3.7	244	n.d.	n.d.	n.d.	16.6	67.0	16.4	1.25
Poppy seed oil (blue seeds)	61 ± 3.7	46	n.d.	214	n.d.	16.9	13.2	69.9	0.36
Poppy seed oil (grey seeds)	51 ± 2.2	56	n.d.	206	n.d.	17.2	11.3	71.5	0.37
Poppy seed oil (white seeds)	68 ± 3.5	39	n.d.	204	n.d.	17.1	11.6	71.3	0.45
Sunflower oil, high content of oleic acid	77 ± 3.3	938	n.d.	n.d.	n.d.	11.4	85.3	3.4	0.18
Sunflower oil	69 ± 2.0	851	23	n.d.	n.d.	10.5	20.5	68.9	0.12
Thistle oil	85 ± 7.4	834	n.d.	n.d.	n.d.	12.1	11.5	76.4	0.96
Walnut oil	89 ± 3.2	35	n.d.	513	56	9.0	15.6	75.4	1.45
Pumpkin seed oil									
1	149 ± 15.8	44	n.d.	603	n.d.	17.5	33.0	49.5	0.37
2	191 ± 17.2	83	n.d.	625	n.d.	21.0	33.7	45.4	0.54
3	166 ± 1.1	18	n.d.	654	n.d.	10.2	41.8	48.0	0.45
4	229 ± 7.1	54	n.d.	711	n.d.	22.6	30.7	46.7	0.41
5	257 ± 15.9	118	n.d.	665	n.d.	21.3	34.3	44.4	0.51
6	255 ± 13.9	110	n.d.	860	n.d.	21.1	35.7	43.2	0.57
7	309 ± 17.5	82	n.d.	801	n.d.	17.3	37.2	45.5	0.84
8	354 ± 19.9	201	n.d.	770	n.d.	17.9	38.9	43.2	1.07
9	348 ± 14.7	120	n.d.	750	n.d.	17.7	37.7	44.6	0.89
Pumpkin seed lipid extracts									
pumpkin seed 1 ($CHCl_3$/MeOH; 1/1)	284 ± 20.2	36	n.d.	460	n.d.	17.8	38.2	44.0	–
($CHCl_3$)	231 ± 10.3	50	n.d.	642	n.d.	17.7	37.5	44.8	–
(*n*-Hexane)	154 ± 10.9	61	n.d.	662	n.d.	16.9	34.8	48.3	–
pumpkin seed 2 ($CHCl_3$/MeOH; 1/1)	305 ± 25.7	9	n.d.	485	n.d.	18.0	38.9	43.1	–
($CHCl_3$)	211 ± 14.3	47	n.d.	593	n.d.	18.1	38.8	43.2	–
(*n*-Hexane)	160 ± 9.5	90	n.d.	529	n.d.	17.8	38.4	43.8	–

[†] Mean values ± standard deviation out of at least three separate measurements.
[‡] Mean values out of at least three separate measurements; standard deviation was always lower than 2%.
[#] Mean values out of at least three separate measurements; standard deviation was always lower than 10%.
[§] n.d. - not detected.

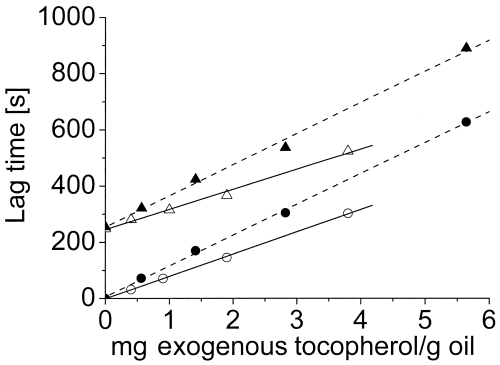

Fig. 3. Effect of exogenously added α-tocopherol (solid line) and γ-tocopherol (dashed line) on lag times of pumpkin seed oil (△; ▲) and pure triacylglycerols (○; ●): Triacylglycerol mixtures were used, corresponding to the triacylglycerol composition of pumpkin seed oil (see Fig. 2). The intrinsic tocopherol contents of the pumpkin seed oil were 110 µg/g (α-) and 860 µg/g (γ-). The apparent lag time of the pumpkin seed oil at 0 mg exogenous tocopherol corresponds to a triacylglycerol sample containing 3190 µg α-tocopherol/g oil or 2270 µg γ-tocopherol/g oil. The correlation parameters are R = 0.9967 (△), R = 0.9975 (▲), R = 0.9998 (○), and R = 0.9989 (●): and P < 0.001 for all graphs.

copherol added to the natural oils led to a further linear increase of the measured lag times. There is the same linear relationship between antioxidant concentration and lag time as already observed for artificial triacylglycerol/tocopherol mixtures (Fig. 3). However, the absolute values of the natural samples were always higher, as a consequence of the contribution of the endogenous tocopherol plus an additional component other than tocopherol. This phenomenon was particularly pronounced in the case of pumpkin seed oils showing the largest lag times of all natural oils in this study.

3.4 Effect of hydrophilic phenolic compounds on lag time

Organic extracts of pumpkin seeds were prepared using solvents or solvent mixtures of different polarity. We found that the more polar extracts led to higher lag times in the fluorescence oxidation assay (Tab. 1). The tocopherol content of the extracts was not responsible for this effect, because it decreased slightly with increasing solvent polarity, while the lag times increased strongly with increasing solvent polarity. As a consequence, the unknown antioxidant compounds must be more polar than tocopherol.

In addition, treatment of pumpkin seed oils with methanol-water also yielded extracts with high antioxidative capacity. When defined portions of these extracts were added to pure triacylglycerol mixtures again an increase in lag times was observed (Fig. 4). In this case, the increase in lag time per unit antioxidant (here: the content in total phenolics) exceeded the antioxidant effect of exogenous tocopherol. Supplementation of pumpkin seed oil with defined portions of these extracts led to an increase of the measured lag times, too, showing the same linear relationship between antioxidant concentration and lag time as already observed for artificial triacylglycerol/tocopherol mixtures (Fig. 4).

Moreover, we carried out experiments with triacylglycerol mixtures containing α-tocopherol, γ-tocopherol or polar extracts, and combinations of these supplements. The samples containing α-tocopherol, γ-tocopherol, or the polar extract showed lag times, which were equal to the sum of the lag times of samples containing only one of the supplements (Tab. 2). In addition, supplementation of pumpkin seed oil with α-tocopherol, γ-tocopherol or polar extracts resulted in the same linear relationship between antioxidant concentration and lag times, as observed for supplementation of triacylglycerol mixtures with these compounds (Fig. 3, Fig. 4). Our data provide clear evidence that under our experimental conditions there is no synergy between the antioxidant effects of α-tocopherol, γ-tocopherol and the polar extract.

From the apparent lag times of natural oils we determined the so-called “non tocopherol-derived lag time“ (nt-lag time) which was defined as the difference of the measured lag time and the lag time corresponding to the total tocopherol content. The nt-lag times correlated best with the concentrations of hydrophilic phenolic compounds, which were determined by the *Folin-Ciocalteu* method

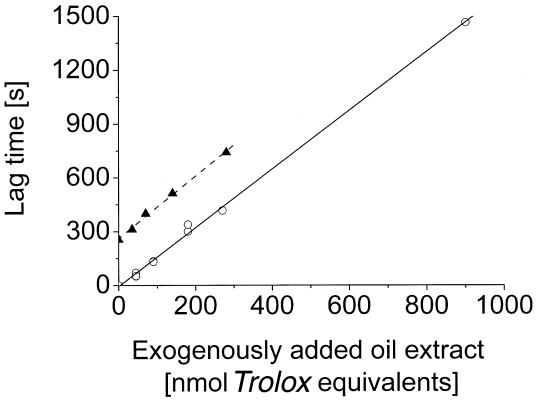

Fig. 4. Effect of exogenously added pumpkin seed oil phenolics on lag times of pumpkin seed oil (▲) and pure triacylglycerols (○): Triacylglycerol mixtures were used containing the same triacylglycerol composition as compared to pumpkin seed oil (see Fig. 2). Lag times are plotted against *Trolox* equivalents of the exogenously added polar extract from pumpkin seed oil (see Materials and methods). The correlation parameters are R = 0.9983 (▲) and R = 0.9989 (○) and P <0.001 for both graphs.

Tab. 2. Measured and calculated lag times for oxidation of triacylglycerols. Effects of tocopherols and polar pumpkin seed extract and mixtures thereof. Measurements were carried out according to Materials and methods. The fatty acid composition of the triacylglycerol mixture was the same as compared to pumpkin seed oil (see legend to Fig. 2).

Tocopherol content		Polar pumpkin seed oil extract	Lag time	
α– [mg/kg]	γ– [mg/kg]	[µmol *Trolox*-equivalents/g lipid]	measured [s]	calculated† [s]
0	0	70	121	–
0.9	0	0	71	–
3.8	0	0	308	–
0	1.4	0	218	–
0.9	1.4	0	284	289
3.8	0	70	430	429
0	1.4	70	343	339

† Calculated on the basis of the samples containing just one antioxidative compound (α- and γ-tocopherol, or polar pumpkin seed oil extract). All measured values are means out of at least three measurements. The standard deviations of the measured lag times were around 10%.

[17] and expressed as *Trolox* equivalents. Good correlations were only found within the same class of oils (Fig. 5). However, such correlations do not exist when different classes of oils are compared. The following relationship was found: the higher the content of hydrophilic phenolic compounds, the higher the nt-lag time. Here, the correlation is shown for pumpkin seed oils because we found the largest effect in this case (Fig. 5).

4 Discussion

The antioxidant content of edible oils is essential for quality and stability. Several methods are available for characterization of oxidizability and antioxidant capacity of oils,

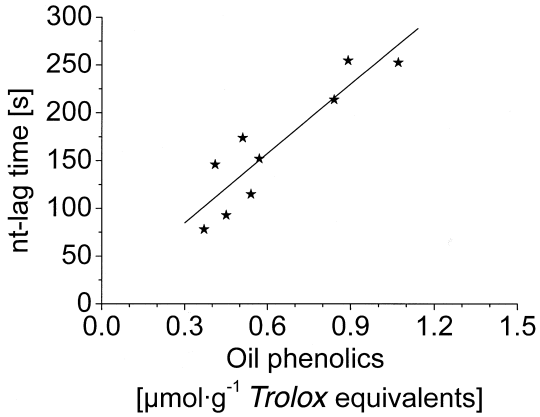

Fig. 5. Correlation between pumpkin seed oil phenolics and antioxidant capacity: Non tocopherol-derived lag times (nt-lag time) for oxidation of pumpkin seed oils were determined as described in Materials and methods and were plotted against *Trolox* equivalents corresponding to the concentrations of oil phenolics. The correlation parameters are R = 0.9097 and P <0.001.

including determination of weight gain, hydroperoxides, peroxide value, TBARS [10] and mass spectroscopic analysis [6] of oxidation products or antioxidants. Many techniques are not convenient for high-throughput screening. Therefore we developed a novel fluorescence technique for this purpose, using fluorescent phosphatidylcholine as a reporter fluorophore (Fig. 1). The latter compound contains a fluorescent fatty acyl chain which shows the same oxidation susceptibility as natural polyunsaturated fatty acids [19] and therefore can be used for reliable measurements of the oxidation of oils and its inhibition by antioxidants. The fluorescence label resides on the surface of the detergent micelles, which were used for oil solubilization in aqueous buffer. As a consequence it is sensitive to antioxidant effects due to lipophilic antioxidants in the lipid droplet and protective effects due to hydrophilic antioxidants in the aqueous phase. The latter compounds represent a first line of defense against free radicals produced by the hydrophilic prooxidants, *e.g.* AAPH or HRP/H_2O_2 which were used in this study. AAPH induces lipid peroxidation by a random flux of peroxyl radicals caused by thermal decomposition. The HRP/H_2O_2 system was used as a biological prooxidant, which generates radicals as a consequence of enzymatic activity.

The relative antioxidant capacities of different tocopherols (α-, β-, γ-, δ-) are extensively described in literature, but the results are contradictory depending on the test system and the assay conditions [9, 20]. Using our new method we found that γ-tocopherol has a 37% higher antioxidant capacity than α-tocopherol. This could be due to the fact that the primary oxidation products of γ-tocopherol may still show antioxidant properties, while those of α-tocopherol do not [21].

Our results showed that edible oils and, in particular, pumpkin seed oil contain at least two classes of effective antioxidant compounds. On the one hand, their antioxidant capacity depends on their content of classical lipophilic antioxidants (*e.g*. tocopherols). On the other hand, we found a major contribution of polar phenolics to the observed antioxidant effects. However, no synergistic effects were observed between α-tocopherol, γ-tocopherol and the polar phenolics. In pumpkin seed oil, 59% of antioxidant capacity is due to polar phenolics and only 41% is due to tocopherols. This assumption is supported by two observations. Firstly, in natural oils containing polar phenolics, we always found a lag time component in addition to the lag time, which had to be expected from the tocopherol content. Secondly, a polar fraction that could be extracted from the oils turned out to be responsible for this effect, since it improved antioxidant capacity to a large extent if it was exogenously added to antioxidant-free triacylglycerol.

Pumpkin seed oil is a natural product, which in contrast to other edible oils is not refined. In this oil, many (antioxidative) components are still preserved that are removed during processing of most other oils. In the latter case, antioxidants (in pure form or as components of unrefined oils) are exogenously added to the oil after processing in order to re-establish a higher antioxidant capacity and as a consequence product quality. It has already been shown that phenolic compounds such as the catechols oleuropein and hydroxytyrosol in virgin olive oil are highly antioxidative [4, 6]. These compounds are polar as well. Our study showed that related substances are responsible for the “nt-lag times” also in other oils, and especially in pumpkin seed oil, where they exert by far the largest effect. A detailed analysis of the phenolic contents of pumpkin seed oil is currently under way in our laboratory. Such information will be extremely useful taking into account the highly beneficial effects which are ascribed to phenolics not only in oils but also in all other foodstuff [6, 18, 22-25]. They might also account for potential health effects of pumpkin seed oil, which so far have only been subject of speculation and are awaiting a critical judgement based on scientific criteria.

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