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DPPH assay of vegetable oils and model antioxidants in protic and aprotic solvents

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ABSTRACT

The rate of reaction of phenolic antioxidants with DPPH depends on solvent composition. The rate constants can differ by more than two orders of magnitude for the same phenolic compound. Reactions are faster in alcohols than in ethyl acetate that is used routinely for the analysis of antioxidant potential (AOP) of nonpolar samples such as vegetable oils. Incorporation of an acid base pair into the assay solvent buffers the system against acid impurities such as free fatty acids and CO_2 from the air. This is shown to increase the rate of oxidation and number of electrons of phenolic compounds exchanged with DPPH. Typically, DPPH assays are performed for predetermined time intervals at which phenolic compounds are not fully oxidized and therefore higher reaction rates result in higher values of AOP. More than twofold AOP was obtained for oleuropein, sesamol, sinapic acid, caffeic acid and protocatechuic acid in buffered alcohols than in ethyl acetate. The AOP of sesame, pumpkin seed and extra virgin olive oil is accordingly higher when determined in buffered alcohols. DPPH assays in ethyl acetate result in underestimation of AOP of unrefined vegetable oils.

1. Introduction

Unrefined vegetable oils are rich sources of antioxidants. They contain tocopherols, tocotrienols, carotenoids and also more polar phenolic compounds that are removed in the refining process [1]. These latter phenolic compounds contribute to the antioxidative potential (AOP) and to sensory properties; unrefined oils accordingly attract a higher price on the market. Caffeic acid, sinapic acid, syringic acid, protocatechuic acid and monohydroxy or dihydroxy phenols such as tyrosol, hydroxytyrosol and sesamol are just some of phenolic compounds that have been identified in methanolic extracts of vegetable oils [2–8] that constitute the so-called hydrophilic fraction (HF).

The AOP of HF containing phenolic compounds [9,10] is typically evaluated by one of the spectrophotometric assays, such as DPPH or ABTS assays, in methanol or aqueous based solvents. AOP in the lipophilic fraction (LF) or the total AOP of vegetable oils is typically determined in ethyl acetate [9] which is sufficiently nonpolar to completely solubilize the lipid matrix. The AOP thus determined is expressed as either the quantity of oil needed to reduce the absorbance of the DPPH radical in the assay by 50% (IC_{50}) [11], the quantity of oil (mg) needed to reduce 0.5 mg of the DPPH radical (EC_{50}) [12], the total free radical scavenging activity (RSC) [9], the relative change in absorbance at fixed added amount of sample (dA_{520}%) [13], or is normalized to the AOP of Trolox, a polar analogue of α-tocopherol [10,14]. The wide variation in the experimental protocols and evaluation of experimental results hinders clarity and consistency of determined AOPs of vegetable oils.

The proticity of the solvent is probably the most important factor defining the reactivity of antioxidants with free radicals. Rates of reaction for a given antioxidant in different solvents can vary by three orders of magnitude [15,16], since the type of solvent defines the reaction mechanism. In aprotic, nonpolar solvents, hydrogen atom transfer from a hydroxyl group prevails [17–19]. In hydrogen bonding solvents, such as alcohols, electron transfer (ET) from the ionized antioxidant becomes the prevalent mechanism and reactions are faster [20]. The rate of reaction can be further increased by incorporation of water [15,21] or, especially, aqueous buffer [22] into the assay solvent.

The practical consequence of the large differences in reaction rate is that DPPH assays, performed for predefined times in different solvents, can lead to large variations in the reported AOP for the same sample. Solvents used for determining the AOP of vegetable oils do not stimulate the ET mechanism, and the reaction of DPPH with antioxidants is therefore slower than in the typical DPPH assay in methanol. This can lead to underestimation of the total AOP with DPPH.

In the present study we have analyzed the influence of solvent composition on the reaction rates of model antioxidants with DPPH (I), and on the reported AOP of vegetable oils and model

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antioxidants at typical incubation times (II). The aim of the investigation was to find a solvent that is sufficiently nonpolar to solubilize vegetable oil and, at the same time to enable a sufficiently high reaction rate of polyphenolic antioxidants with DPPH to result in a more correctly determined AOP.

2. Experimental

2.1. Chemicals

2,2-diphenyl-1-picrylhydrazyl (D9123; DPPH), (±)-6-hydroxyl-2,5,7,8-thetramethylcromane-2-carboxylic acid (238813; Trolox), (±)-α-tocopherol (T3251), 3,4-methylenedioxyphenol (S3003; sesamol), 3,5-dimethoxy-4-hydroxycinnamic acid (D7927; sinapic acid), 3,4-dihydroxybenzoic acid (37580; protocatechuic acid), 3-(3,4-dihydroxyphenyl)-2-propenoic acid (C0625; caffeic acid), (4S,5E,6S)-4-[2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl]-5-ethylidene-6-[[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)-2-tetrahydropyranyl]oxy]-4H-pyran-3-carboxylic acid methyl ester (O8889; oleuropein), and water for trace analysis (95305 Fluka) were obtained from Sigma–Aldrich (Steinheim, Germany).

Palmitic acid (P0500), methanol (1.06009), propan-2-ol (1.09634), propan-1-ol (1.00997), ethyl acetate (1.09623), acetic acid (1.00063), Tris buffer (1.08382; (hydroxymethyl)aminomethane) and KCl (1.04938) were obtained from Merck (Darmstadt, Germany).

Extra virgin olive oil (EVOO), pumpkin seed oil (PSO), sesame oil (SEO), rapeseed oil (RSO), linseed oil (LSO), sunflower oil (SUO) were purchased on the Slovenian market.

2.2. Solvent preparation

DPPH assays were performed in ethyl acetate (EA), methanol (M), mixtures of methanol and propan-2-ol 1:1 (V/V) (MP) and MP containing the acid–base pair tris(hydroxymethyl)aminomethane (Tris, 30.0 mM) and acetic acid (37.8 mM) in molar ratio 1:1.26 (MP-AB). Tris is more readily soluble in methanol than in MP mixture, therefore the stock solution for MP-AB was prepared in methanol. Solutions were prepared daily and used fresh. Older solutions of Tris and acetic acid in methanol led to lower stability of DPPH in the control solution.

Dielectric properties of solvents were determined by the capacitive method, using a Precision Liquid Test Fixture 16452A and a precision LCR meter E4980A (both Agilent Technologies) connected to a personal computer. Dielectric parameters were measured at 75 frequencies between 20 Hz and 2 MHz at 25.0 °C ± 0.1 °C. Temperature was controlled by a high precision water bath 7320 (Fluke) and external thermometer system 5627 A/1502 A (Fluke).

The dielectric constant (ε') was determined at 2 MHz frequency as described by Midmore et al. [23]. Electrolytic conductivity (κ) was determined using a standard solution of KCl in a mixture of 30% propan-1-ol in water for trace analysis, according to Wu et al. [24]. Resistance (R) was plotted as a function of frequency^{-1} and the extrapolated value of R at frequency$^{-1}=0$ used to calculate κ.

Measurements of dielectric constant for pure, freshly opened solvents (Table 1) correspond to literature data [25,26]. Values of ε' and κ, determined in MP mixture, correspond to the average values of the pure solvents. The dielectric constant did not change significantly (only 4% increase), when an acid–base pair was incorporated into the mixture of methanol and propan-2-ol. Much bigger differences in conductivity were observed where κ values were changed by three orders of magnitude due to ionization, since ionic species (protonated Tris, acetate) contribute to higher conductivities.

2.3. DPPH assay – determination of rate constants

The kinetics of reaction of model phenolic compounds with DPPH, that has a high molar extinction coefficient at 520 nm, were recorded on a Cary 100 UV–vis spectrophotometer with its temperature controller set to 25 °C. Stock solutions of DPPH in methanol, DPPH radical in ethyl acetate and model phenolic compounds in methanol (300 μM) were prepared daily and stored in the dark at 25 °C until use.

Assay solutions were prepared by mixing the appropriate volumes of stock solutions of DPPH, pure solvents, Tris base and acetic acid in methanol (where applicable) directly in a quartz cuvette to give a total volume of 2950 μL, sealed with a cap and incubated in a thermostated cuvette holder for 10 min. The reaction was started by adding 50 μL of 300 μM solution of antioxidant, mixing thoroughly and immediately replacing in the cuvette holder. The final concentration of DPPH was 100 μM and of the antioxidants 5 μM. Absorbance at 520 nm was measured for 3 min in alcoholic solvents and for 2 h in ethyl acetate. The reaction of model antioxidant with DPPH results in the formation of $DPPH_2$ that has low absorbance at 520 nm.

Reactions were performed under pseudo-first order conditions at large molar excess of DPPH over antioxidants (molar ratio 20:1). The kinetics were best fitted as a single, first order reaction (Eq. (1)) with program Origin 8.

$$A_t = A_1 \cdot e^{-t \cdot k_{obs}} + A_\infty \quad (1)$$

where A_t is the absorbance at 520 nm at time t and A_1 is the decrease in absorbance (dA_{520}) of the first phase of oxidation of antioxidants with DPPH. dA_{520} for all antioxidants was 0.11 ± 0.01, which corresponds to the exchange of two electrons of antioxidant with DPPH (5 μM antioxidant; $\varepsilon_{DPPH\ 520\ nm} \approx 11\ 000\ L\ mol^{-1}\ cm^{-1}$; 1 cm cuvette; $\varepsilon_{DPPH2\ 520\ nm} \approx 0$). A_∞ is the offset (absorbance at 520 nm after the end of first phase). k_{obs} (min^{-1}) is the rate constant. In order to compare our results with those of others the second order rate constants were calculated (Eq. (2)).

$$k_{obs} = k \cdot [DPPH] \Rightarrow k = \frac{k_{obs}}{[DPPH]} \quad (2)$$

Table 1
The dielectric constants (ε') and electrolytic conductivities (κ) of solvents used in the DPPH assay.

Solvent	*M*		*P*		EA		MP		MP-AB	
	ε'	κ (μS cm^{-1})	ε	κ (μS cm^{-1})	ε'	κ (μS cm^{-1})	ε'	κ (μS cm^{-1})	ε'	κ (μS cm^{-1})
Freshly opened solvents	32.7	1.08	19.48	0.03	6.31	< 0.001	25.67	0.43	26.65	800
Solvents exposed to air	32.84	1.28	19.42	0.04	n.d.[c]	n.d. [c]	25.99	0.87	27.12	800
Literature data	32.66[a]	0.0015[a]	19.25[a]	0.06[a]	6.02[a]	< 0.001[a]	n.d.[c]	n.d.[c]	n.d.[c]	n.d. [c]
	32.63[b]		19.72[b]		6.05[b]					

[a] [25].
[b] [26].
[c] Not determined.

and

$$t_{95\%} = -\frac{\ln(0.05)}{k_{obs}} \quad (3)$$

where $t_{95\%}$ (min) is the time in which 95% of the first phase amplitude is achieved (Eq. (3)). We have defined this as the time needed for quantitative oxidation in the first phase.

All experiments were performed in triplicate. Standard deviations of determined rate constants are within 10%.

2.4. DPPH assay – influence of acid impurities on the oxidation of protocatechuic acid

We have determined whether CO_2 from the air or the free fatty acids normally present in vegetable oils have an influence on the reactivity of protocatechuic acid in MP and MP-AB with DPPH. As a control freshly opened methanol and propan-2-ol were used. When studying the influence of free fatty acids, experiments were performed in solvents containing 100 mg/L palmitic acid; for the influence of atmospheric CO_2, the solvents were left in glass flasks open to air for 24 h prior to the assay. Palmitic acid was chosen because it is the most common saturated fatty acid in vegetable oils. Water content in solvents was determined by the volumetric Karl Fischer method. Kinetic experiments were performed with protocatechuic acid (5 μM) and DPPH (100 μM) at 25 °C. Results are presented as relative change in absorbance at 520 nm. Absorbances at 520 nm in each solvent were normalized to the absorbance at $t=0$. The kinetic curves presented in each solvent are the average of three experiments.

2.5. DPPH assay – antioxidant potential of model phenolic compounds and vegetable oils

AOPs of selected model phenolic compounds and vegetable oils were determined by measuring the absorbance of the samples and blanks (only DPPH) at 520 nm at pre-chosen time intervals: 30, 60, 120 300 and 1440 min. For the analysis of vegetable oils, 100 mg of oil samples were weighed into 15 mL screw-cap polypropylene centrifuge tubes and dissolved in 9.900 mL of DPPH. For the analysis of model antioxidants, 100 μL of stock solution of model antioxidant in methanol was mixed with 9.900 mL of DPPH in appropriate solvents. For blanks, 100 μL of methanol was mixed with 9.900 mL of DPPH in appropriate solvents. All solvents and centrifuge tubes were incubated at 25 °C prior to the assay. After mixing solutions were kept at 25 °C in the dark. Aliquots of 1 mL were transferred from each centrifuge tube into polypropylene cuvettes at pre-chosen time intervals and absorbances at 520 nm measured immediately. Results are presented as $dA_{520,}$ calculated as the difference in the absorbance of the blanks (only DPPH) and samples/model antioxidants, all measured at pre-chosen time intervals. All experiments were performed in triplicate, including weighing of oil samples and model phenolic compounds for preparation of stock solutions.

3. Results and discussion

3.1. Rate constants of the reaction of model phenolic compounds with DPPH are smaller in ethyl acetate than in alcohols

Solvent composition has a large influence on the rate of oxidation of model phenolic compounds by DPPH for the initial oxidation step (Table 2). The amplitude of that step corresponds to the exchange of two electrons for one molecule of antioxidant [19,27].

In ethyl acetate, which is used routinely for the assaying the AOP of vegetable oils, the reaction rates for all model antioxidants are slower in comparison to values determined in protic solutes (Table 2). In absolute terms, Trolox and α-tocopherol gave the highest rates. Here, quantitative oxidation with DPPH (95% of the amplitude) was achieved within 10 min. Typical incubation times for the analysis of vegetable oils with DPPH are longer, indicating that AOP of refined oils containing predominantly vitamin E analogues can be correctly evaluated in this solvent. The reaction rates of caffeic acid, sezamol and protocatechuic acid, that are present in unrefined vegetable oils, are lower than those for vitamin E analogues by more than an order of magnitude, only being oxidized quantitatively after a few hours. Previously reported rate constants for the oxidation of α-tocopherol with DPPH are comparable to the value determined in our study [28]. Since caffeic acid, sesamol and protocatechuic acid are more polar than vitamin E analogues, the kinetics are normally analyzed in alcohols and no published data about their reactivity in ethyl acetate are available.

The rates of reaction of all analyzed phenolic compounds with DPPH in methanol are higher than in ethyl acetate (Table 2). Rate constants for the oxidation of protocatechuic and caffeic acids are more than 300-fold higher, whereas the smallest (threefold) difference was observed for α-tocopherol. The kinetic constants shown in Table 2 are in accordance with published data for vitamin E analogues [27–29], protocatechuic acid [27] and caffeic acid [19,27,30].

The rate of oxidation has also been measured in a mixture of methanol and propanol-2-ol. This mixture is sufficiently nonpolar to solubilize up to 3% of triacylglycerols, which is a precondition when vegetable oils are analyzed, and on the other hand sufficiently polar to solubilize the Tris:acetate base pair. Rate constants for caffeic acid and vitamin E analogues are higher in the presence of the acid base pair (Table 2), whereas no acceleration was observed for sesamol and protocatechuic acid. This is in accordance with previous experiments, where we showed that the presence of an acid–base pair in the solvent results in higher reactivity of some polyphenols with DPPH [22]. In the presence of base, rates are even higher, however, deprotonated $DPPH_2$

Table 2
Pseudo-first order rate constants, and time needed to complete 95% of the amplitude of the first phase, for the reaction of phenolic compounds (5 μM) with DPPH (100 μM) in alcohols and ethyl acetate at 25 °C.

Phenolic compound	*M*		MP		MP-AB		EA	
	k_{obs} (min^{-1})	$t_{95\%}$ (min)	k_{obs} (min^{-1})	$t_{95\%}$ (min)	k_{obs} (min^{-1})	$t_{95\%}$ (min)	k_{obs} (min^{-1})	$t_{95\%}$ (min)
Protocatechuic acid	1.0	2.9	1.0	3.0	1.0	2.9	0.0028	1100
Sesamol	2.1	1.5	2.0	1.5	2.0	1.5	0.0067	450
Caffeic acid	5.6	0.54	6.1	0.49	11	0.28	0.018	170
α-Tocopherol	2.3	1.3	2.1	1.4	6.7	0.45	0.38	7.9
Trolox	4.9	0.62	4.0	0.74	9.1	0.33	0.29	11

Values are the average of three determinations and the standard deviation was ca. ± 10%.

($DPPH^-$) has a higher absorbance at 520 nm, which limits the applicability of the spectrophotometric assay at basic pH [31].

3.2. An acid–base pair in the assay solvent buffers against acidic impurities and increases the extent of oxidation of protocatechuic acid with DPPH

Acids have a large influence on the reactivity of polyphenols with DPPH, starting in the micromolar range [32], so we sought to see whether impurities such as CO_2 from the air or free fatty acids transferred with the sample of vegetable oil have an influence on the reaction rates in the DPPH assay. Protocatechuic acid, for which the slowest rate in the initial phase was observed, was analyzed in the mixture of alcohols with and without acid–base pair.

In the absence of the acid–base pair, addition of palmitic acid (100 μg/mL) resulted in the decrease of reaction rate by approximately one order of magnitude (Fig. 1). These are the conditions that are encountered in the analysis of AOP of vegetable oils. Unrefined oils typically contain 1% of free fatty acids [33] and a 100-fold dilution into the assay solvent results in a concentration similar to that above. A reaction rate lower than that in the control was also observed in solvents equilibrated with air prior to the assay (Fig. 1). Purging solvents with pure CO_2 resulted in a further decrease of reaction rates (results not shown). If this mixture was purged with N_2 prior to the assay, reaction rates were higher (Fig. 1), which is a strong indication that CO_2 itself contributed to the lower reaction rates. When the assays were performed in solutions purged with N_2, the rates were slightly higher than in the control. This can be ascribed to the accumulation of water from the air, known to increase the reaction rates. The control solution contained 0.18 mg/mL of H_2O and that equilibrated with air, 0.45 mg/mL. The higher water content is also reflected in the higher conductivity and dielectric constant of the solvent exposed to air in open flasks for 24 h (Table 1). Acid impurities and water undoubtedly have a large influence on the course of reaction. This fact is often neglected when AOP of antioxidants is evaluated with DPPH.

The acid–base pair has practically no influence on the reaction rate of the fast initial phase (Table 2 and Fig. 1). The large difference of rates from those in the assay in the absence of the acid–base pair is observed for secondary reactions. Oxidation of polyphenols is a complex, multistep process. The partially oxidized polyphenols that are formed initially can be further oxidized [28,30,34]. The amplitudes of these slower phases are quantitatively very important [28] and can contribute substantially to the AOP determined in typical DPPH assays. These secondary reactions proceed much faster in the presence of an acid–base pair, and the amplitude (dA_{520}) after 30 min is much larger than in a pure mixture of alcohols (Fig. 1). When experiments are performed in the presence of an acid–base pair, acid impurities and traces of water influence the reaction rates minimally, differences are statistically insignificant. The course of reaction in the presence of 100 mg/L palmitic acid is the same as in the control solution (Fig. 1).

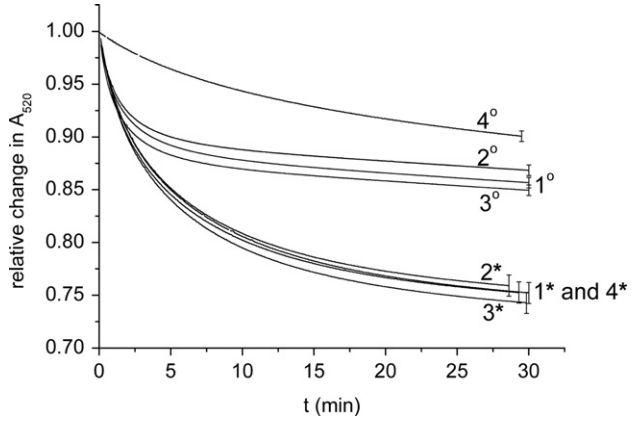

Fig. 1. Influence of acid impurities on the reaction of protocatechuic acid with DPPH in MP (°) and MP-AB (∗).The experiments were performed with freshly opened solvents (1); with solvents left open to air for 24 h (2); with solvents that, after 24 h exposure to air, were purged with nitrogen (3); and with freshly opened solvents in the presence of 100 mg/L palmitic acid (4). Standard deviations of three experiments for endpoint dA_{520} under each condition are shown with error bars.

3.3. Solvent composition has a large influence on the antioxidant potentials determined for model phenolic compounds and vegetable oils

3.3.1. The absorbance of DPPH at 520 nm in the presence of model antioxidants and vegetable oils measured at predetermined time intervals

When AOP of a complex mixture is determined, samples are usually incubated with DPPH for 30–120 min before measuring the absorbance in the range 515–520 nm [35]. We have shown that the rate of reaction of some antioxidants with DPPH is slow in ethyl acetate (Table 2). The reaction is significantly faster when the assay is performed in alcohols and incorporation of the acid–base pair results in higher reactivity of partially oxidized protocatechuic acid with DPPH (Fig. 1). For this reason we have analyzed the influence of solvent composition (EA, MP, MP-AB) and the change in A_{520} that is directly proportional to the AOP.

When incubation is prolonged, the stability of DPPH determines the applicability of the assay of antioxidants with slow reaction rates. Its stability in MP-AB is less than in EA in which, after 24 h, the absorbance at 520 nm decreases by less than 5% (Table 3). In MP-AB a similar decrease was observed after only 1 h of incubation, which is in the range of stability of DPPH in methanol [22,36].

Some of the model antioxidants that have been identified in unrefined oils [2–8] and vegetable oils were analyzed at the same time intervals as for DPPH alone. For Trolox only minor differences in dA_{520} were determined in all three solvents after 30 min incubation. The molar ratio Trolox:DPPH, calculated from dA_{520}, $\varepsilon_{DPPH\ 520\ nm}$ and the concentration of Trolox in the assay, is close to 1:2 in all three solvents as described previously [22,27]. During prolonged incubation only minor changes in dA_{520} were observed in EA, whereas in MP-AB and MP it decreased gradually. This can be ascribed to the instability of DPPH, since the same relative decrease in absorbance of the control and of the sample results in a smaller dA_{520}, due to the larger absolute change of A_{520} in the control.

For α-tocopherol, practically the same dA_{520} was observed after 30 min in all three solvents, as for its structural analogue Trolox, resulting in the same molar ratio antioxidant:DPPH as reported [37]. However, in contrast to Trolox, a time dependent increase of dA_{520} in EA, accounting for more than two exchanged electrons, was observed. This can be explained by the more complex pattern of oxidation of α-tocopherol in EA as shown by the different products identified by HPLC-MS analysis of oxidized α-tocopherol [38,39].

EA is not an appropriate solvent for the analysis of AOP of polyphenolic antioxidants. dA_{520} for each antioxidant determined in MP-AB after 30 min is larger than that determined in EA after 300 min (Table 3). An assay in MP gives a dA_{520} comparable to that in MP-AB only for sesamol. The AOP of samples as unrefined vegetable oils containing polyphenols like oleuropein and caffeic, synapic and protocatechuic acids can therefore be significantly underestimated when assaying in alcoholic solvents or EA after typical incubation times for DPPH assay. Incorporation of the acid–base pair into the

alcohols influence the reactivity of the quinones, formed in the fast oxidation step. For protocatechuic acid it is most likely the result of higher reactivity of quinones towards nucleophilic attack of the methanol. Methoxy group attached to the aromatic ring results in the regeneration of catechol (benzene-1,2-diol) structure that can be reoxidized with DPPH resulting in additional number of exchanged electrons. Regeneration of catechol structure and no oxidation by DPPH is the rate limiting step in methanol [40].

The low reactivity of polyphenolic antioxidants in EA and MP is also reflected in AOPs determined for EVOO, PSO and SEO. Those oils are rich sources of polyphenolic antioxidants and higher dA_{520} values were observed in MP-AB. In contrast, for sunflower oil (SUO) that contains predominantly vitamin E analogues as antioxidants [9,41], larger values of dA_{520} were obtained in EA at all chosen time intervals. This is in accordance with the more extensive oxidation of α-tocopherol by DPPH observed in EA (Table 3).

3.3.2. *Polyunsaturated fatty acids react with DPPH*

When oils were analyzed in EA a large increase in dA_{520} was observed after prolonged incubation that could not be attributed solely to the additional oxidation of vitamin E analogues. This was most pronounced for linseed oil, where all DPPH was exhausted after 300 min and for PSO, SEO and RSO oils after 24 h incubation (Table 3). It has been shown that conjugated linoleic acid is very reactive in the DPPH assay [42]. Since it is predominant in animal fat [43] we have determined whether linoleic and linolenic acids, that are major polyunsaturated fatty acids (PUFA) in vegetable oils [43,44], also react with DPPH in EA and in MP-AB (Appendix A, Supplementary data). Initially, the rates of oxidation are similar in the two solvents and, to some degree, contribute to the determined AOP. At longer incubation times, reactions are faster in EA, in which all the DPPH (initial concentration 100 μM) is completely exhausted after 180 min in the presence of 100 mg/L of linolenic acid, and after 300 min in the presence of 100 mg/L linoleic acid. The long incubation times that

Table 3
Influence of incubation time on the quenching of DPPH by antioxidants and oil samples.

Sample	Concentration	Solvent	30 min	60 min	120 min	300 min	1440 min
Control (only DPPH)	100 μmol/L	**EA**	1.109	1.111	1.114	1.098	1.063
		MP	1.081	1.066	1.056	1.005	0.857
		MP-AB	1.066	1.038	1.019	0.961	0.792
Trolox	20 μmol/L[a]	**EA**	0.479	0.477	0.476	0.468	0.457
		MP	0.464	0.458	0.449	0.418	0.332
		MP-AB	0.438	0.428	0.417	0.385	0.334
α-Tocopherol	20 μmol/L[a]	**EA**	0.465	0.493	0.520	0.541	0.527
		MP	0.440	0.432	0.420	0.399	0.312
		MP-AB	0.430	0.410	0.397	0.369	0.266
Caffeic acid	15 μmol/L[a]	**EA**	0.123	0.211	0.279	0.356	0.364
		MP	0.357	0.369	0.345	0.350	0.418
		MP-AB	0.516	0.578	0.648	0.703	0.741
Sinapic acid	15 μmol/L[a]	**EA**	0.151	0.174	0.216	0.239	0.306
		MP	0.278	0.284	0.296	0.294	0.321
		MP-AB	0.335	0.376	0.432	0.457	0.454
Sesamol	20 μmol/L[a]	**EA**	0.112	0.187	0.275	0.378	0.397
		MP	0.391	0.420	0.429	0.447	0.467
		MP-AB	0.388	0.384	0.384	0.396	0.446
Protocatechuic acid	15 μmol/L[a]	**EA**	0.011	0.028	0.049	0.093	0.233
		MP	0.228	0.287	0.367	0.498	0.658
		MP-AB	0.671	0.720	0.739	0.728	0.710
Oleuropein	20 μmol/L[a]	**EA**	0.144	0.221	0.285	0.324	0.334
		MP	0.324	0.324	0.324	0.329	0.369
		MP-AB	0.549	0.624	0.655	0.659	0.636
Extra virgin olive oil (EVOO)	10 g/L[b]	**EA**	0.238	0.259	0.268	0.293	0.514
		MP	0.244	0.237	0.231	0.266	0.264
		MP-AB	0.390	0.440	0.492	0.580	0.668
Pumpkin seed oil (PSO)	10 g/L[b]	**EA**	0.272	0.297	0.332	0.373	0.983
		MP	0.305	0.309	0.306	0.311	0.318
		MP-AB	0.335	0.373	0.383	0.400	0.465
Sesame oil (SEO)	10 g/L[b]	**EA**	0.240	0.311	0.357	0.425	1.030
		MP	0.285	0.303	0.333	0.355	0.434
		MP-AB	0.402	0.385	0.411	0.443	0.510
Rapeseed oil (RSO)	10 g/L[b]	**EA**	0.359	0.409	0.453	0.510	1.043
		MP	0.373	0.366	0.360	0.372	0.373
		MP-AB	0.392	0.392	0.396	0.403	0.392
Linseed oil (LSO)	10 g/L[b]	**EA**	0.303	0.328	0.354	0.995	1.024
		MP	0.298	0.297	0.284	0.299	0.396
		MP-AB	0.327	0.336	0.346	0.350	0.418
Sunflower oil (SUO)	10 g/L[b]	**EA**	0.320	0.349	0.376	0.419	0.500
		MP	0.250	0.233	0.204	0.182	0.150
		MP-AB	0.247	0.238	0.231	0.208	0.160

[a] Concentration of the antioxidant in the assay medium.
[b] Concentration of vegetable oil in the assay medium.

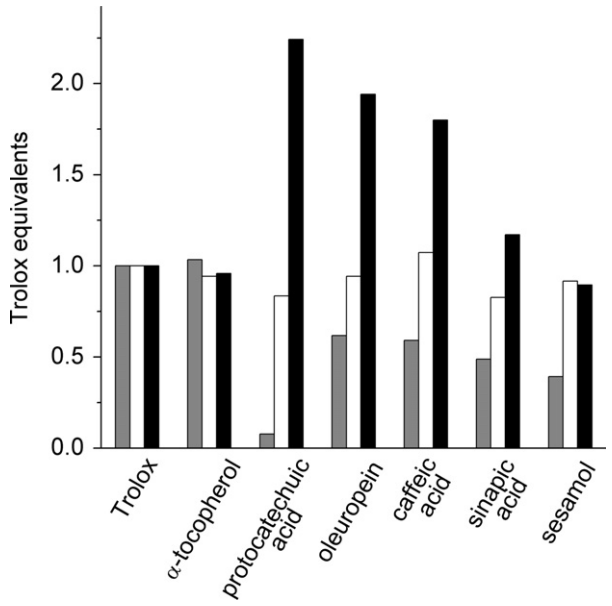

Fig. 2. Influence of solvents on the stoichiometry of the reaction of model antioxidants with DPPH. The resulting dA_{520} (1 h at 25 °C) in EA (gray), in MP (white) and in MP-AB (black) were normalized to the concentration and dA_{520} determined for Trolox in same solvents. Concentrations of antioxidants and DPPH in the assay were the same as shown in Table 3.

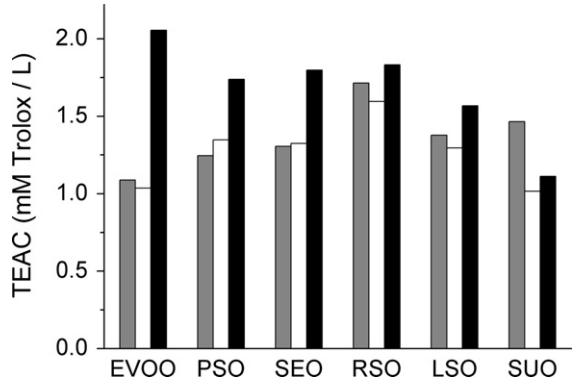

Fig. 3. Influence of solvents on the antioxidant potential (AOP) of vegetable oils determined with DPPH. The resulting dA_{520} (1 h at 25 °C) in EA (gray), in MP (white) and in MP-AB (black) were normalized to the concentration and dA_{520} determined for Trolox in same solvents. The results are expressed as Trolox equivalent antioxidant capacity (TEAC) in the oils (mM Trolox/L). The experimental details and abbreviations used are the same as shown in Table 3.

would be needed to fully account for the AOP of polyphenols in EA are therefore inappropriate and could result in great overestimation of AOP due to the presence of PUFA in vegetable oils.

The instability of DPPH in MP and MP-AB, and the reactivity of PUFA in EA can result in experimental artifacts when evaluating AOP after prolonged incubation. In order to minimize such effects and to compare our results with the published work [9–11,13,45], a 1 h incubation was chosen for presentation of results normalized to Trolox equivalents (TE) for model antioxidants (Fig. 2) and Trolox equivalent antioxidant capacity (TEAC) for oil samples (1 TEAC = 1 mM Trolox/L) (Fig. 3).

3.3.3. *Trolox equivalents of model antioxidants and the antioxidant potential of vegetable oils for the reaction with DPPH in protic and aprotic solvents after 1 h incubation*

For α-tocopherol, 1.1 TE was obtained in EA, whereas AOP in MP and MP-AB is equimolar to Trolox. AOP determined for model polyphenols depends to a large extent on the solvents used for the assay. In EA, the time of incubation is too short to account even for the "fast phase" where two electrons (1 TE) are exchanged. In MP, AOP values are higher than in EA but, with the exception of sesamol, lower than in MP-AB. The largest difference was observed for protocatechuic acid, present in some vegetable oils [7,9], for which an almost 30-fold greater AOP was determined in MP-AB than in EA. The contribution of benzoic acids, such as protocatechuic acid, to AOP is therefore completely neglected when analysis is carried out in solvents like EA. The influence of solvent on values of AOP of caffeic acid and oleuropien, also present in vegetable oils [9] and having 3,4-hydroxyl groups on the aromatic ring, is similar. The reactions of partially oxidized polyphenols [30] contribute to values of AOP only in MP-AB (Fig. 2).

The influence of solvent composition on values of AOP of vegetable oils is not unambiguous. Of the six oils analyzed, those that contain a substantial amount of relatively polar phenolic compounds, such as extra virgin olive oil [2,8] and cold pressed pumpkin seed oil [6], gave higher AOP values when analyzed in MP-AB. For EVOO an almost twofold greater value of AOP was observed in MP-AB than in the assays in two other solvents. For sesame oil, in which the major antioxidant is the phenolic compound sesamol [5], similar to cold pressed pumpkin seed oil, a 1.4-fold greater value of AOP was obtained in MP-AB than in EA. For sunflower oil, which is practically devoid of polyphenols [10] and whose major antioxidants are vitamin E analogues [2,9], a more than 1.3-fold greater AOP was obtained in EA than in MP-AB.

Model antioxidants, that are more polar than tocopherols, can be extracted from vegetable oils into methanol [9] or mixtures of methanol and water [10]. In order to confirm that AOP is underestimated when assayed in EA, due to more polar polyphenols, we extracted polar antioxidants from EVOO with a mixture of methanol and water (80:20 V/V) in a volume ratio 1:1, and analyzed the separated fractions with DPPH in EA and in MP-AB. A large difference in dA_{520} measured after 60 min incubation was observed for the polar fraction after 100-fold dilution into DPPH solutions. In EA, dA_{520} was only 0.082 ($\pm$0.006) and in MP-AB 0.201 ($\pm$0.014). In methanol, that is routinely used for assessing AOP of polar antioxidants in oils [9], dA_{520} (0.141 $\pm$ 0.006) was substantially smaller than in MP-AB. Such results are in accord with the reactivity of model polyphenols in various solvents (Fig. 2). For the nonpolar fraction, analyzed under the same conditions, dA_{520} in EA was 0.190 ($\pm$0.009) and, in MP-AB, 0.234 ($\pm$0.015). The fact that a larger dA_{520} was obtained in MP-AB can be attributed to incomplete extraction of polar polyphenols, since two or three cycles with polar solvent are usually applied for quantitative extraction of polyphenols from oil [10]. The sum of values of dA_{520} for the polar and nonpolar fractions, determined in both EA and MP-AB, corresponds to the dA_{520} determined for EVOO after 60 min incubation (Table 2) in each of two solvents, indicating that there is no synergism in AOP of polar and nonpolar antioxidants.

3.3.4. *Comparison of the antioxidant potentials of vegetable oils*

The antioxidant potential of vegetable oils has been determined using DPPH in several studies. In the original papers AOP was expressed as dA_{520}% [13], IC_{50} [11], RSC [9] or TEAC [10,45]. We have normalized all results to TEAC, assuming that the molar ratio Trolox: DPPH is 1:2 and that the molar extinction coefficient of DPPH is 11 000 L mol^{-1} cm^{-1}. The length of the assays, the temperature and the concentration of antioxidants in oils differed in those experiments, so comparison of the results with each other or with those of our study is uncertain. The fivefold span in determined AOP (Table 4) for EVOO is therefore not surprising. Koprivnjak et al. [13] (TEAC = 1.2) and Jiang et al. [45] (TEAC = 1.1) also found that AOP for EVOO in ethyl acetate is close to 1 TEAC. Torres et al. [12] found that AOP for EVOO in toluene is 1.5 TEAC. Only the results of Espin et al. [9] stand out from the others,

Table 4
Antioxidant potentials (AOP) of vegetable oils expressed as Trolox equivalent antioxidant capacity in oils (mM Trolox/L) as determined in various laboratories.

AOP of oil	EVOO	PSO	SEO	LSO	RSO	SUO
Our study	1.1[a] (2.1[b])	1.2[a] (1.7[b])	1.3[a] (1.8[b])	1.4[a] (1.6[b])	1.7[a] (1.8[b])	1.5[a] (1.1[b])
Tuberoso et al. [10]	1.3[a]	1.0[a]	n.d.	1.0[a]	0.8[a]	1.1[a]
Espin et al. [9]	2.7[a]	n.d.[c]	7.8[a]	8.1[a]	9.4[a]	7.5[a]
Rossi et al. [11]	0.5[a]	n.d.[c]	n.d.[c]	n.d.[c]	n.d.[c]	1.5[a]

[a] Determined by DPPH in ethyl acetate.
[b] Determined by DPPH in a mixture (50:50 V/V) methanol: propanol-2-ol containing 30 mM Tris and 38 mM acetic acid.
[c] Not determined.

not only for EVOO but also for other oils. According to the latter authors, antioxidants are found predominantly in the lipophilic fraction, where vitamers of vitamin E are rarely present in total concentrations higher than 1 mM [41]. The possibility that our calculations of TEAC were wrong cannot be excluded, but it is more likely that polyunsaturated fatty acid contributed to the AOP determined by Espin et al. [9], since the relative scavenging activity was determined from kinetic equations by extrapolation of experimental data. The AOP values for vegetable oils from other studies are comparable to that determined for AOP in EA in our study, and substantially smaller than that measured in MP-AB.

4. Conclusions

There is no straightforward answer to the question as to how to perform DPPH assays of vegetable oils. For refined vegetable oils, where practically no polyphenols are present, ethyl acetate could be the solvent of choice. Oxidation of tocopherol is slower in EA than in alcohols but still sufficiently fast, since two electrons are exchanged within minutes (Table 2). In the oxidation of alpha-tocopherol in EA even more than two electrons are exchanged, which contributes to the higher value of AOP for sunflower oil after 60 min than in alcohols (Table 3). With this incubation time, linolic and linolenic acids do not contribute significantly to the determined AOP.

For unrefined oils such as EVOO, sesame and pumpkin seed oil, where not only vitamin E vitamers, but also more polar polyphenols contribute to the AOP, EA is not an appropriate solvent, due to the slow and incomplete oxidation of polyphenols. Incorporation of an acid base pair into the alcohols has been shown to buffer the medium against acids, such as atmospheric CO_2 or free fatty acids from the matrix. This significantly increases the rate of oxidation and number of electrons of phenolic compounds exchanged with DPPH. AOP of unrefined vegetable oils is accordingly more correctly determined in buffered alcohols (Fig. 3).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.03.046.

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