European Food Research and Technology (2019) 245:1473–1485
https://doi.org/10.1007/s00217-019-03255-7

ORIGINAL PAPER

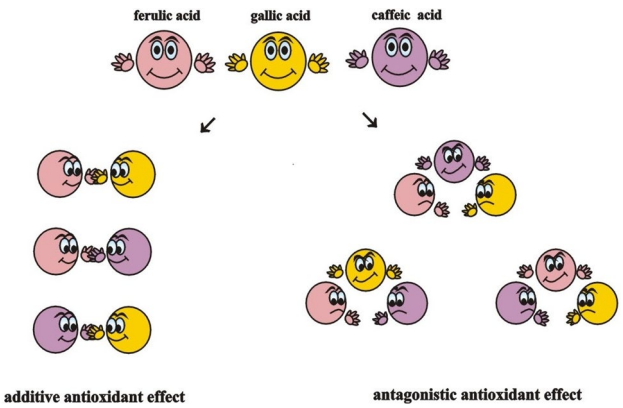
Are mutual interactions between antioxidants the only factors responsible for antagonistic antioxidant effect of their mixtures? Additive and antagonistic antioxidant effects in mixtures of gallic, ferulic and caffeic acids

Małgorzata Olszowy[1] · Andrzej L. Dawidowicz[1] · Małgorzata Jóźwik-Dolęba[2]

Received: 6 December 2018 / Revised: 18 January 2019 / Accepted: 9 February 2019 / Published online: 26 February 2019

Abstract
The antagonistic effect of antioxidant properties among various mixtures of phenolic substances is the subject of numerous works and inquiries. The present study shows and discusses the antioxidant properties of binary and ternary mixtures of the chosen phenolic compounds in which additive and antagonistic antioxidant effects are observed. Gallic, ferulic and caffeic acids were applied in the experiments as model phenolic antioxidants. The antioxidant properties of these compounds and their mixtures were estimated by the ABTS method in aqueous and ethanol/aqueous solutions. The presented data proved that the observed antioxidant antagonism in the mixtures of the examined antioxidants does not result from the mutual interactions between individual mixture components but from the difference in reaction kinetics between a given antioxidant and the ABTS cation radical. The magnitude of the observed antagonism depends on mutual relations of individual components and solvent type.

Graphical abstrsct



Keywords Phenolic acids · Antagonistic antioxidant effect · Additive antioxidant effect · Food components

Abbreviations

ABTS 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

✉ Małgorzata Olszowy
malgorzata.olszowy@poczta.umcs.lublin.pl

[1] Faculty of Chemistry, Maria Curie Sklodowska University, Pl. Marii Curie Sklodowskiej 3, 20-031 Lublin, Poland

[2] Pollena-Aroma, Przemysłowa 14, 05-100 Nowy Dwór Mazowiecki, Poland

Introduction

The interest in the impact of nutritional and pro-healthy components on the proper functioning of living organisms results in the fact that functional food represents one of the most intensively investigated and widely promoted areas in the food and nutrition sciences today. Special attention with respect to design of functional foods is paid to the application of food components exhibiting antioxidant properties which commonly occur in the form of mixtures in nature. As results from the literature report, the antioxidant properties of antioxidant mixtures are not always the additive value of individual mixture components [1, 2]. The experimentally observed antagonistic and synergistic antioxidant effects in various antioxidant mixtures are the subject of numerous papers and inquiries [3–5]. Numerous papers point out that the observed non-additive antioxidant effects in the antioxidant mixtures result from mutual interactions between their individual components. Yet, the exact causes have not been elucidated.

The aim of this study is to explain the reasons for the antagonistic antioxidant effect observed experimentally in the multicomponent antioxidant mixtures. The paper reports and discusses the antioxidant properties of three phenolic compounds; gallic, ferulic and caffeic acids, and their binary and ternary mixtures. The popularity of these acids results not only from their prevalence in nature but also from their pro-health anti-inflammatory, anticancer, antifungal, and antibacterial properties [6–10], which predestinate their application as functional food components.

The ABTS assay regarded as a direct, rapid, simple and reliable method of estimating antioxidant activity was applied in the experiments.

Materials and methods

Reagents

Gallic acid, ferulic acid and caffeic acid, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate (di-potassium peroxdisulfate) and methanol for HPLC were purchased from Sigma-Aldrich (Poznań, Poland). Ethanol came from the Polish Factory of Chemicals—Avantor Performance Materials Poland S.A. (Gliwice, Poland). Water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA).

Preparation of antioxidant solutions

Standard solutions of gallic acid (0.015 mg/ml), ferulic acid (0.02 mg/ml) and caffeic acid (0.002 mg/ml) in water, ethanol and ethanol/water mixtures containing 15% or 40% of ethanol were prepared. The concentrations of the phenolic acids applied in the experiments correspond to the natural levels of these compounds in wines. The antioxidant properties of the individual components and of their binary and ternary mixtures were examined. The volume ratios of antioxidant standards solutions and antioxidant standard solvents for binary and ternary mixtures are presented in Tables 1 and 2, respectively.

Methods

ABTS assay

Generation of the ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] radical cation was performed by Nenandis [11]. The $ABTS^{\bullet+}$ solution was prepared by the reaction of 5 ml of a 7 mM aqueous ABTS solution and 88 µl of 140 mM (2.45 mM final concentration) potassium persulfate ($K_2S_2O_8$). The mixture was incubated in the dark for 16 h [12]. The radical cation formed in this way was diluted in water or ethanol or ethanol/water mixture containing 15% or 40% of ethanol until the initial absorbance value of 0.8 (for data obtained in Figs. 1, 2, 3, 4) or 0.4; 0.5 or 1.0 (for data in Figs. 5, 6, 7) at 744 nm was reached. 2 ml of $ABTS^{\bullet+}$ solution was mixed in a 4 ml test tube with 100 µl (see Table 1) or 90 µl of the antioxidant/solvent or antioxidant mixture (see Table 2) or 100 µl of mixture composed of 33 µl of the examined antioxidant (range $c = 0.0$–0.02 mg/mL) and 66 µl of ethanol/water mixture containing 15% or 40% of ethanol (for data in Figs. 6, 7). The mixture was stirred vigorously for 30 s and poured into quartz cuvettes (1 cm × 1 cm × 3.5 cm). The decrease in absorbance was monitored at the wavelength of 744 nm for 30 min at 25 °C. The absorption measurements were recorded using a UV Probe-1800 Spectrophotometer (Shimadzu, Kyoto, Japan).

To zero the spectrophotometer, water or ethanol or ethanol/water mixture containing 15% or 40% of ethanol was used.

The percent of inhibition was calculated from the following equation:

$$I(\%) = \left(1 - \frac{A_t}{A_{t_0}}\right) \times 100\%,$$

Table 1 Volumes of gallic, ferulic and caffeic acid solutions used for the estimation of the antioxidant properties of these compounds and their binary mixtures

Components	Sample number																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Gallic acid in solvent* (µl)	20	40	60	80	100	–	–	–	–	–	–	–	–	–	–	20	40	60	80	–	–	–	–	80	60	40	20
Ferulic acid in solvent* (µl)	–	–	–	–	–	20	40	60	80	100	–	–	–	–	–	80	60	40	20	20	40	60	80	–	–	–	–
Caffeic acid in solvent* (µl)	–	–	–	–	–	–	–	–	–	–	20	40	60	80	100	–	–	–	–	80	60	40	20	20	40	60	80
Solvent* (µl)	80	60	40	20	–	80	60	40	20	–	80	60	40	20	–	–	–	–	–	–	–	–	–	–	–	–	–
Total volume (µl)	100																										

*Water or ethanol or ethanol/water mixture containing 15 or 40% of ethanol

where A_{t_0} and A_t are the values of $ABTS^{\bullet+}$ absorbance at 0 min and at time equal to (t) min.

The expected antioxidant activities were calculated by the addition of the experimentally estimated antioxidant activities for individual antioxidants (the precise way of calculation was described in "Results and discussion" in description of Figs. 1a, 4).

HPLC measurements

The quantitative estimation of the examined phenolic compounds after the reaction with ABTS radical cation was carried out by HPLC. All measurements were performed using a Gilson with UV–Vis detector, a fluorescence detector (Jasco) and an ODS column (Microsorb MV 100 C18, 15 cm × 4.6 mm i.d.). The sample components were eluted using the following elution program: 0–15 min isocratic elution (5% B) and then gradient of B (5–100%) from 15 to 80 min. Water with acetic acid (5% solution in water) and methanol played the role of solvent A and B, respectively. The samples were injected with a sample injector (Rheonyne 7725) equipped with a 20 µl loop. A wavelength of 254 nm was used for the UV–Vis detector, whereas the fluorescence measurements were performed at $\lambda_{ex} = 278$ nm and $\lambda_{em} = 366$ nm over 7.0 min in the case of gallic acid, and then (after 7 min) at $\lambda_{ex} = 260$ nm and $\lambda_{em} = 420$ nm in the case of ferulic and caffeic acids.

Statistical analysis

The results are presented as mean values. To determine the measurements' reproducibility, each antioxidant activity assay was repeated three times. RSD of all the measurements were lower than 10%. $P < 0.05$ was assumed as the statistical difference between the experimental points. Comparisons between experimental and expected antioxidant properties for binary and ternary antioxidant systems were made by the modified Student's t test. All statistical analyses were performed using the Statistica version 7.0 software package (Statsoft, Tulsa, USA) [12].

Results and discussion

Figures 1, 2 and 3 present the influence of gallic or ferulic or caffeic acid amount in one-component solutions and in their binary mixtures (see Table 1), all differing in solvent type:

- water—Figs. 1a, 2a and 3a;
- ethanol/water mixture containing 15% of ethanol—Figs. 1b, 2b and 3b;
- ethanol/water mixture containing 40% of ethanol Figs. 1c, 2c and 3c or

Table 2 Volumes of gallic, ferulic and caffeic acid solutions used for the estimation of the antioxidant properties of these compounds and their ternary mixture

Components	Sample number															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Gallic acid in solvent* (µl)	–	–	–	30	45	15	–	–	–	30	45	30	15	45	15	30
Ferulic acid in solvent* (µl)	–	–	–	–	–	–	45	30	15	45	30	30	45	15	30	15
Caffeic acid in solvent* (µl)	15	30	45	–	–	–	–	–	–	15	15	30	30	30	45	45
Solvent* (µl)	75	60	45	60	45	75	45	60	75	–	–		–	–	–	–
Total volume (µl)	90															

*Water or ethanol or ethanol/water mixture containing 15 or 40% of ethanol

- ethanol—Figs. 1d, 2d and 3d, on the antioxidant activity of the measuring system.

The dashed line with triangles in Figs. 1 and 2 represents gallic acid; the dash–dotted line with circles in Figs. 2 and 3 corresponds to ferulic acid, whereas the dotted line with stars in Figs. 1 and 3 is for caffeic acid. Figures 1, 2 and 3 also contain the expected curves (dotted lines with diamonds) constructed by figuring out the experimental individual antioxidant activities of the examined antioxidants. At the bottom of each figure, there are three axes which help to relate individual experimental points in the figures with sample numbers listed in Table 1. The concentration of each antioxidant was expressed by the volume of its solution in a 100 µL sample introduced to the measuring system. For better understanding of the results, four points are marked in Fig. 1a: a–d.

Point "a" corresponds to inhibition percent of the measuring system containing 80 µL of gallic acid aqueous solution and 20 µL of water in 100 µL sample (sample number 4 in Table 1).

Point "b" corresponds to inhibition percent of the measuring system containing 20 µL of caffeic acid aqueous solution and 80 µL of water in 100 µL sample (sample number 11 in Table 1).

Point "c" corresponds to inhibition percent of the measuring system containing 20 µL of caffeic acid and 80 µL of gallic acid (both as aqueous solutions) in 100 µL sample (sample number 24 in Table 1).

Point "d" is the so-called "theoretical" point representing inhibition percent expected for the mixture containing of 80 µL of gallic acid and 20 µL of caffeic acid, assuming antioxidant effect additivity. The value was calculated by figuring out the inhibitions percent shown by the sample composed of 80 µL of gallic acid and 20 µL of solvent (see point "a") and by the sample composed of 20 µL of caffeic acid and 80 µL of solvent (see point "b").

As results from the presented figures, the curves corresponding to the individual components reflect the evident relationship for antioxidants: their concentration increase (expressed as volume) causes the growth of inhibition percent of a given antioxidant. However, most important for the present data is to compare the experimentally estimated antioxidant activity of binary mixtures with their expected antioxidant activity calculated from the data for the individual antioxidants (solid and dotted line, respectively). As results from the figure, the run of the experimental and the expected curves is almost the same. In all cases, the observed differences are statistically insignificant ($p > 0.05$) which indicates that the antioxidant properties of antioxidant binary mixtures are a sum of antioxidants properties of their antioxidant components. This finding is consistent for all the used solvents (see a–d parts of Figs. 1, 2, 3), i.e., the additive antioxidant effect of gallic/ferulic or gallic/caffeic or ferulic/caffeic acid mixtures on the scavenging process of ABTS cation radicals is observed in all of them.

A more detailed consideration of the results presented in Figs. 1, 2 and 3 shows that the antioxidant activity of the examined acids and their binary mixtures depends also on the composition of the reaction environment. For all the acids and acid pairs, the greatest antioxidant activity is observed in the ethanol/water mixture containing 40% of alcohol (see part c in Figs. 1, 2, 3). This fact can be connected with the viscosity of the ethanol/water mixture, which is the greatest for the 40% ethanol solution. It cannot be excluded that the most viscous ethanol/water structure exhibits the highest proton and electron transmission and, consequently, is responsible for the highest antioxidant properties of the examined phenolic acids. The importance of solvent type in the estimation of antioxidant activity is known from the literature [13, 14]. Moreover, Dawidowicz and Olszowy [15] showed that, the water content in antioxidant solvent has also a distinct impact on the inhibition percent, which is the measure of antioxidant activity. Hence, some visible differences in the antioxidant activity of the examined acids and their binary mixtures in water, ethanol and water/ethanol solvents are understandable.

While the additive antioxidant effect in binary mixtures has been explained for the situation when both antioxidants have the same antioxidant efficiency [16], these answers are not helpful when the three examined acids exhibit different antioxidant power, gallic acid being the most powerful antioxidant. In light of the data from Figs. 1, 2 and 3, the

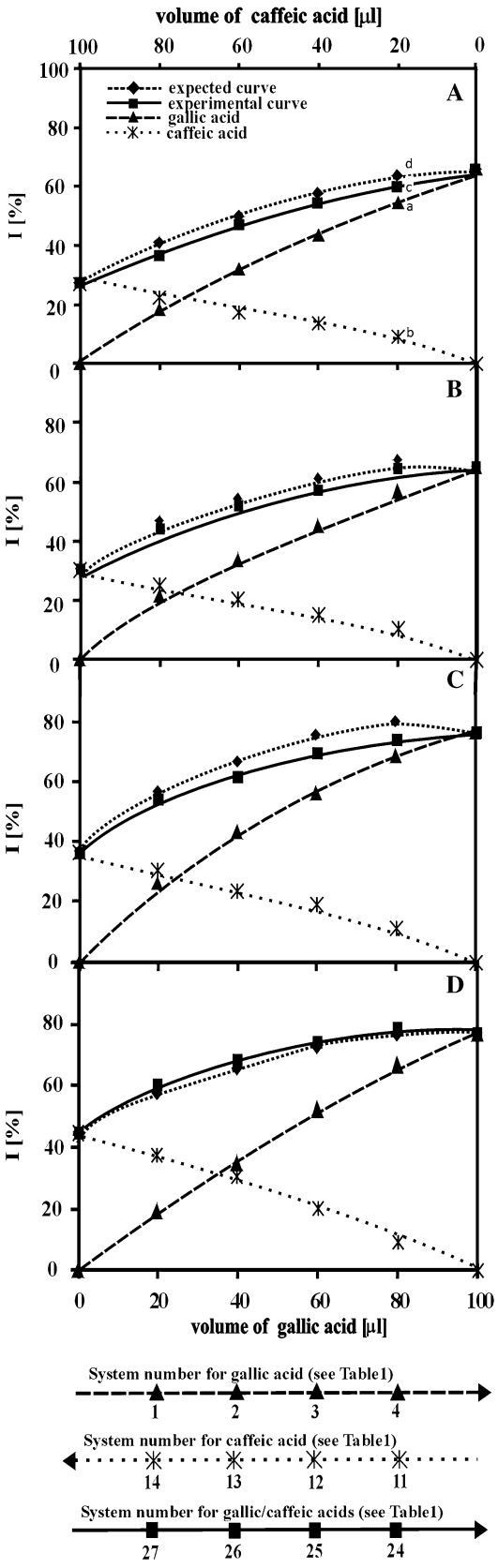


Fig. 1 The antioxidant activity changes for systems containing different volumes of: gallic acid solution (dashed line with triangles), caffeic acid solution (dotted line with stars), gallic and caffeic acid solutions (solid line with squares), estimated by ABTS assay in water (**a**) or water/ethanol, 85/15 v/v (**b**) or water/ethanol, 60/40 v/v (**c**) or ethanol, 96% (**d**). Experimental values are mean values for $n=3$. Dense dotted line with diamonds corresponds to the expected values for gallic and caffeic acid solutions. Volume compositions of the examined solutions are given in Table 1. At the bottom of the figure, for clarity, additional axes with the examined sample numbers (see Table 1) were introduced

observation of Aoun and Makris does not apply to all experimental systems.

Subsequent experimental steps involved ternary mixtures. Figure 4 presents the antioxidant activity, expressed as inhibition percent of ABTS cation radicals, for caffeic acid (bars with dots), gallic acid (bars with chequered pattern), ferulic acid solutions (white bars) and for their ternary mixtures (bars with diagonal strips) differing in the amounts of individual components and estimated as different in solvent type:

- water—Fig. 4a;
- ethanol/water mixture containing 15% of ethanol—Fig. 4b;
- ethanol/water mixture containing 40% of ethanol Fig. 4c or
- ethanol—Fig. 4d

The number of each bar in a given set of bars corresponds to the number of examined samples listed in Table 2, e.g.:

- bar number 1 reflects the antioxidant activity of sample 1 from Table 2 composed of 15 µL of caffeic acid solution and 75 µL of a given solvent;
- bar number 4 reflects the antioxidant activity of sample 4 from Table 2 composed of 30 µL of gallic acid solution and 60 µL of a given solvent;
- bar number 7 reflects the antioxidant activity of sample 7 from Table 2 composed of 45 µL of ferulic acid solution and 45 µL of a given solvent;
- bar number 10 reflects the antioxidant activity of sample 10 from Table 2 composed of 15 µL of caffeic acid solution, 30 µL of gallic acid solution and 45 µL of ferulic acid solution, all in the same solvent;

In these experiments, all the samples introduced to the measuring systems were 90 µL.

The set of bars in Fig. 4 contains also the expected antioxidant activities constructed by adding up the experimental activity data for each examined antioxidant (black bars labelled "Σ")—e.g., bar labelled Σ_a represents the anticipated inhibition percent of the mixture containing 15 µL of caffeic acid, 30 µL of gallic acid and 45 µL of ferulic

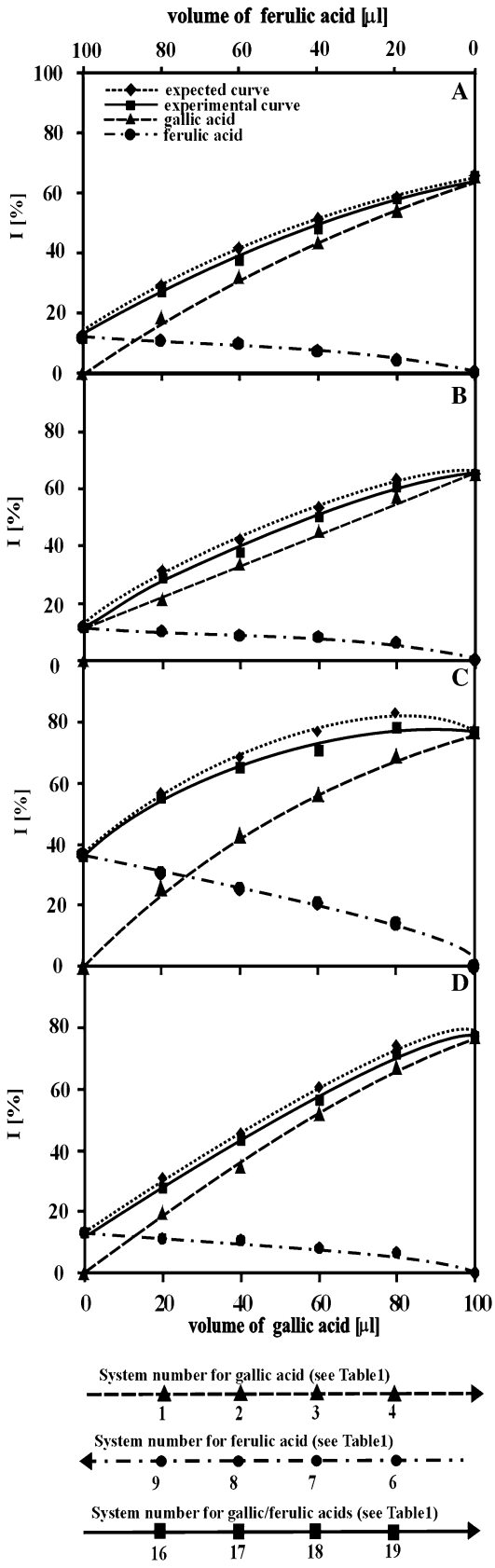


Fig. 2 The antioxidant activity changes for systems containing different volumes of: gallic acid solution (dashed line with triangles), ferulic acid solution (dash–dotted line with circles), gallic and ferulic acid solutions (solid line with squares), estimated by ABTS assay in water (**a**) or water/ethanol, 85/15 v/v (**b**) or water/ethanol, 60/40 v/v (**c**) or ethanol, 96% (**d**). Experimental values are mean values for $n=3$. Dense dotted line with diamonds corresponds to the expected values for gallic and ferulic acid solutions. Volume compositions of the examined solutions are given in Table 1. At the bottom of the figure, for clarity, additional axes with the examined sample numbers (see Table 1) were introduced

acid assuming antioxidant effect additivity. The value was calculated by figuring out the inhibitions percent shown by samples 1, 4 and 7.

The analysis of the results shows that the expected antioxidant properties of ternary mixture are greater than that experimentally determined. The significance of the difference between the experimental and expected antioxidant activity of ternary mixtures has been estimated by the T and p values listed in Table 3. The higher the T value is, the more significant the difference will be. As results from the analysis of the data from Table 3, the observed difference is insignificant ($p>0.05$) only for sample no. 13 (see bars labelled 2 6 7 Σ_d 13 in ethanol in Fig. 4d). Thus, the obtained experimental data generally indicate the antagonistic antioxidant effect of the ternary mixtures of the examined acids on the scavenging process of free radicals. The magnitude of this effect depends on the mutual concentration relations among individual components (expressed by the volumes of the antioxidant solutions) and solvent types (see Fig. 4; Table 3).

It is very difficult to account for the observed phenomenon as the antagonism and synergism of antioxidants have not yet been described in detail. It has been suggested in the literature that antagonism among antioxidants can result from:

- regeneration of a less effective antioxidant by a more effective antioxidant [17, 18];
- oxidation of a more effective antioxidant by the radicals of a less effective antioxidant [17, 18];
- competitive formation of antioxidant adducts [19–22], and
- alteration of the microenvironment of one antioxidant by another antioxidant [17, 23].

To answer which of these reasons is most probably responsible for the observed antioxidant antagonism in the examined ternary mixtures, the analyses of measuring systems containing single antioxidants and their binary and ternary mixtures were performed using HPLC with mass spectrometry and fluorescence detection. These experiments excluded the formation of adducts, dimers and/or oxidative

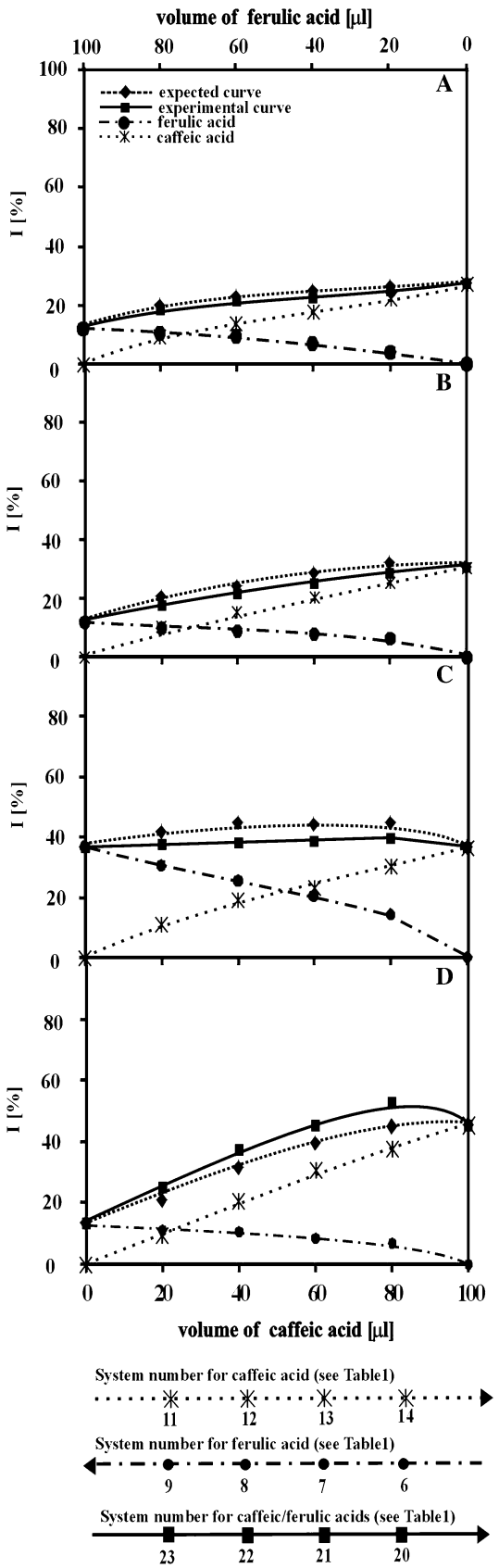


Fig. 3 The antioxidant activity changes for systems containing different volumes of: caffeic acid solution (dotted line with stars), ferulic acid solution (dash–dotted line with circles), caffeic and ferulic acid solutions (solid line with squares), estimated by ABTS assay in water (**a**) or water/ethanol, 85/15 v/v (**b**) or water/ethanol, 60/40 v/v (**c**) or ethanol, 96% (**d**). Experimental values are mean values for $n=3$. Dense dotted line with diamonds corresponds to expected values for caffeic and ferulic acid solutions. Volume compositions of the examined solutions are given in Table 1. At the bottom of the figure, for clarity, additional axes with the examined sample numbers (see Table 1) were introduced

products of antioxidants and did not show increased consumption of a stronger antioxidant at the presence of a weaker one (which would indicate the regeneration process of the weaker antioxidant by the stronger one). The only evident observation in these experiments was a clear consumption decrease of ferulic acid (the weakest antioxidant used) in ternary mixtures in relation to its consumption in the measuring systems containing only this antioxidant and its mixture with gallic or caffeic acid. This observation is supported by the experimental data from Tables 4 and 5 showing depletion of ferulic acid in the measuring systems containing only this antioxidant and its binary (Table 4), and its ternary (Table 5) mixtures with gallic and/or caffeic acid. A more detailed analysis of the results from Table 5 reveals additionally an equivocal relation between the depletion of ferulic acid (the weakest antioxidant used) and the concentration of gallic acid (the strongest antioxidant used) in the measuring system: the greater the gallic acid concentration in the ternary mixture of the antioxidant, the smaller the depletion of ferulic acid. This relation may support the validity of the hypothesis assuming the regeneration of a less effective antioxidant by a more effective one [17, 18]. According to Rúa et al. [18], antioxidant antagonism in binary mixtures of antioxidants results from the difference in the reduction potential of individual components. However in light of the literature data concerning the reduction potential of the examined phenolic acids, the validity of this explanation is difficult to accept. According to Rúa et al. [18] and Chen et al. [24], the reduction potentials of these compounds are similar. Hence, the lack of antioxidant antagonism in binary mixtures of the examined antioxidants (Figs. 1, 2, 3) seems to be plausible. On the other hand, the applicability of this theory for the explanation of the observed antagonisms in the ternary mixtures is disputable. There are a few papers [25, 26] reporting different reduction potentials for the examined antioxidants—lower for gallic and caffeic, and higher for ferulic acid. In such a case, the regeneration theory would be helpful to explain the antagonistic antioxidant effect observed in ternary mixtures, but would not be sufficient for binary mixtures containing ferulic acid, for which additivity of antioxidant properties of individual components is observed—see Figs. 2 and 3.

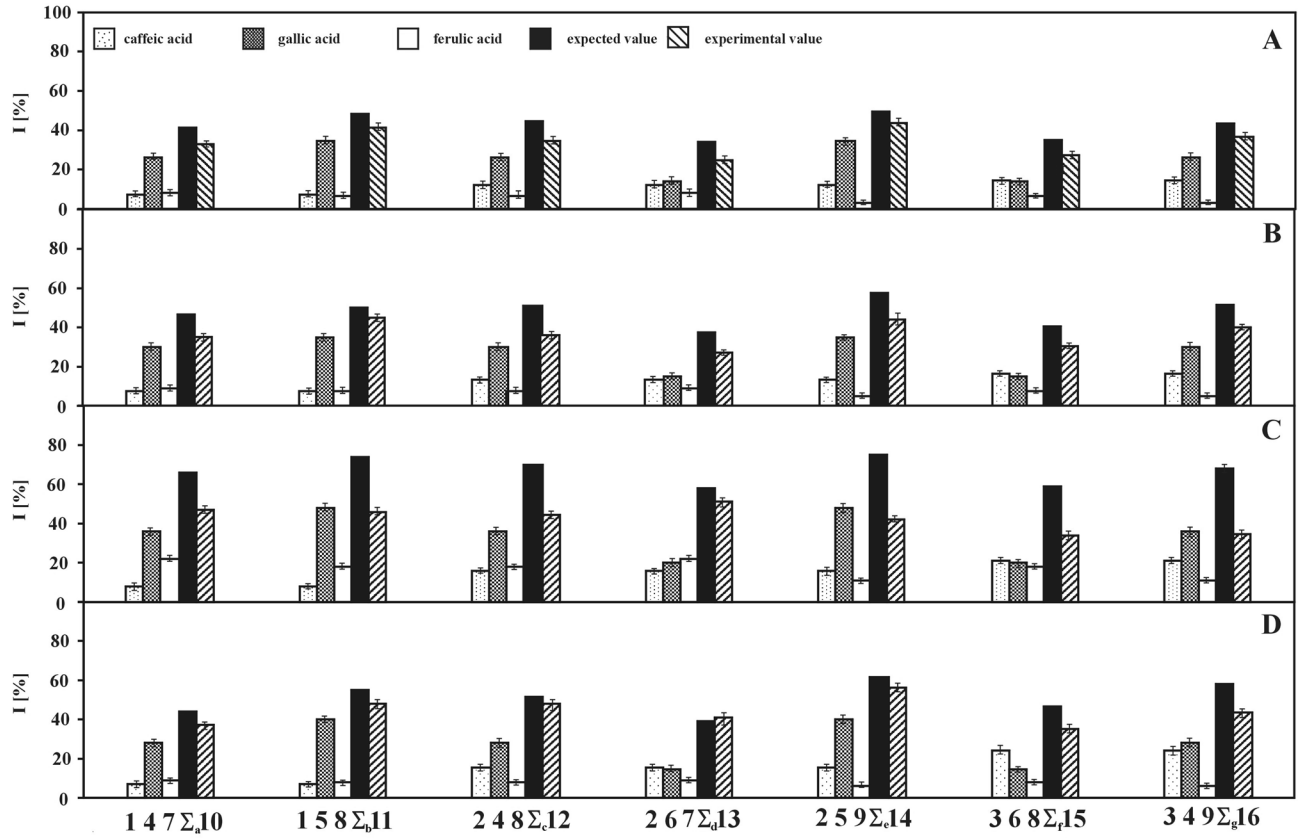


Fig. 4 The antioxidant activity changes for systems containing different volumes of: caffeic acid solution (bars with dots), gallic acid solution (bars with chequered pattern), ferulic acid solution (white bars), caffeic and gallic and ferulic acid solutions (bars with diagonal strips), estimated by ABTS assay in water (**a**) or water/ethanol, 85/15 v/v (**b**) or water/ethanol, 60/40 v/v (**c**) or ethanol, 96% (**d**). Black bars labelled as "Σ" correspond to expected values of antioxidant activity for given ternary system. The numbers of bars correspond to the numbers of samples from Table 2. Experimental values are mean values for $n = 3$

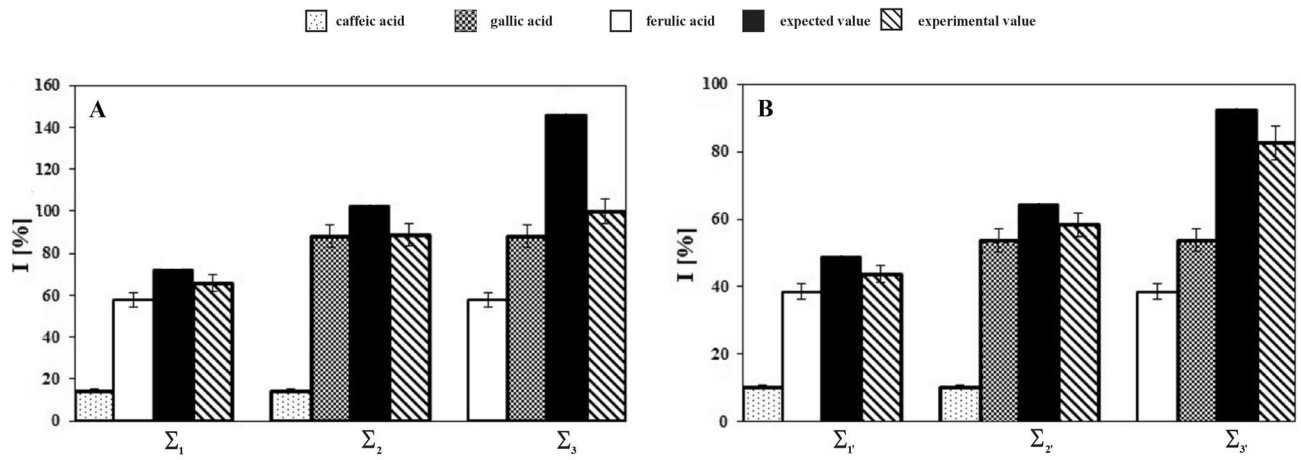


Fig. 5 The antioxidant activity changes for systems containing equal volumes of: caffeic acid solution (bars with dots), gallic acid solution (bars with chequered pattern), ferulic acid solution (white bars), caffeic and gallic and ferulic acid solutions (bars with diagonal strips), estimated by ABTS assay in 40% of ethanol. Black bars labelled as "Σ" correspond to the expected values of antioxidant activity for binary systems. Experimental values are mean values for $n = 3$

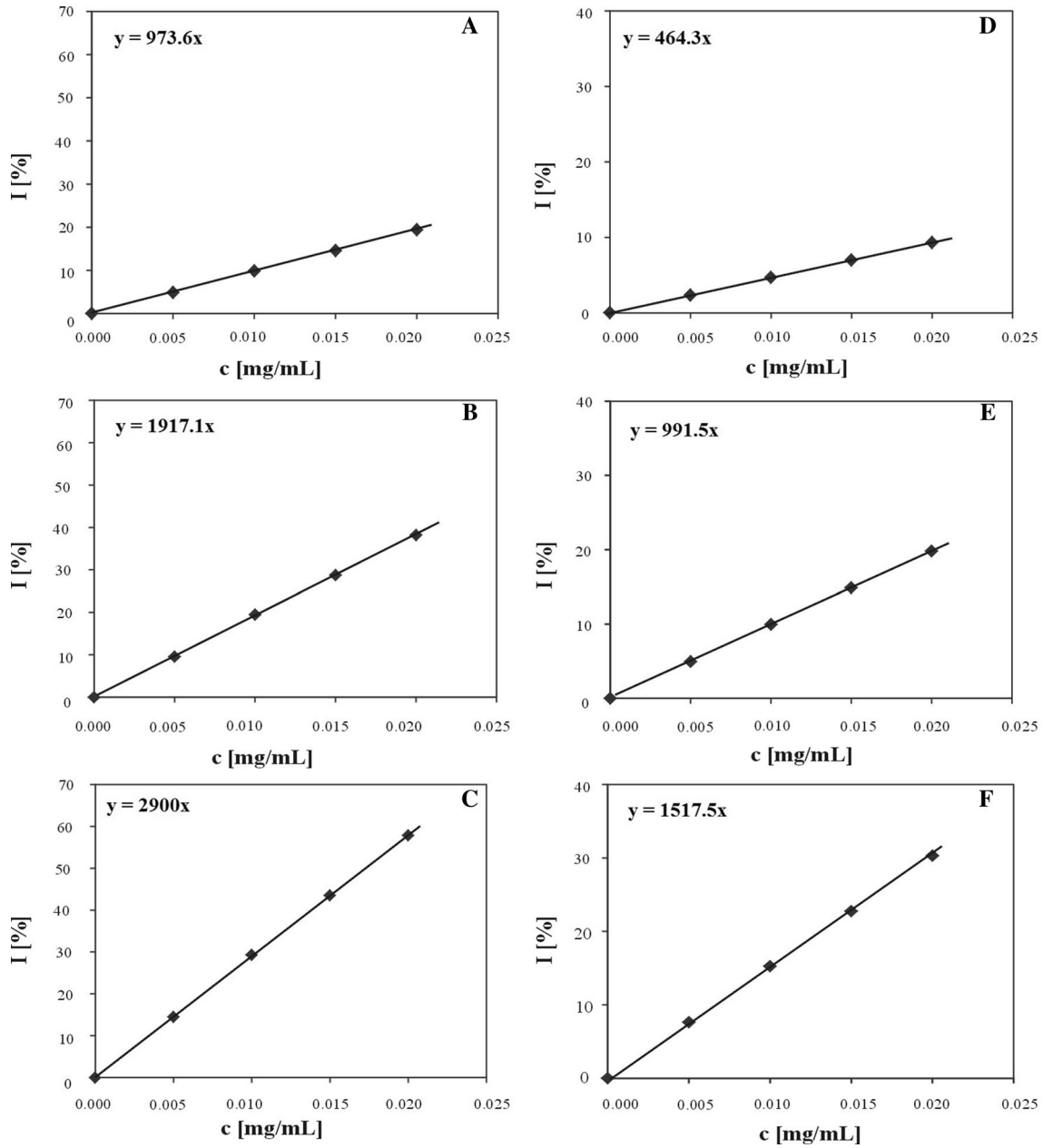


Fig. 6 The antioxidant activity changes for systems containing different concentrations of: ferulic acid solution (**a, b**), caffeic acid solution (**c, d**), gallic acid solution (**e, f**), estimated by ABTS assay in water/ethanol, 85/15 v/v at absorbance of cation radical equals 0.5 (**a, c, e**) and 1.0 (**b, d, f**). Experimental values are mean values for $n = 3$

Tables 6 and 7 show the depletions of gallic acid in measuring systems containing only this antioxidant and its binary and ternary mixtures with ferulic and/or caffeic acid. As results from these data, the total depletion of gallic acid is observed, independently on the examined system. These data and those from Tables 4 and 5 suggest another possible explanation of the antagonistic effect in antioxidant ternary mixtures: differences in the reaction kinetics between a given antioxidant and the ABTS cation radical. The strongest antioxidant quickly reduces the ABTS cation radical concentration, thus decreasing the reaction rate between the weaker antioxidant and the radicals due to the quick lowering concentration of the latter. If this is true, the magnitude of the observed antagonistic effect in mixtures of the examined antioxidants should depend not only on antioxidant concentrations and solvent type (confirmed by the data in Fig. 4) but also on the cation radical concentration.

Figure 5 presents the antioxidant activity, estimated in 40% of ethanol and expressed as inhibition percent of

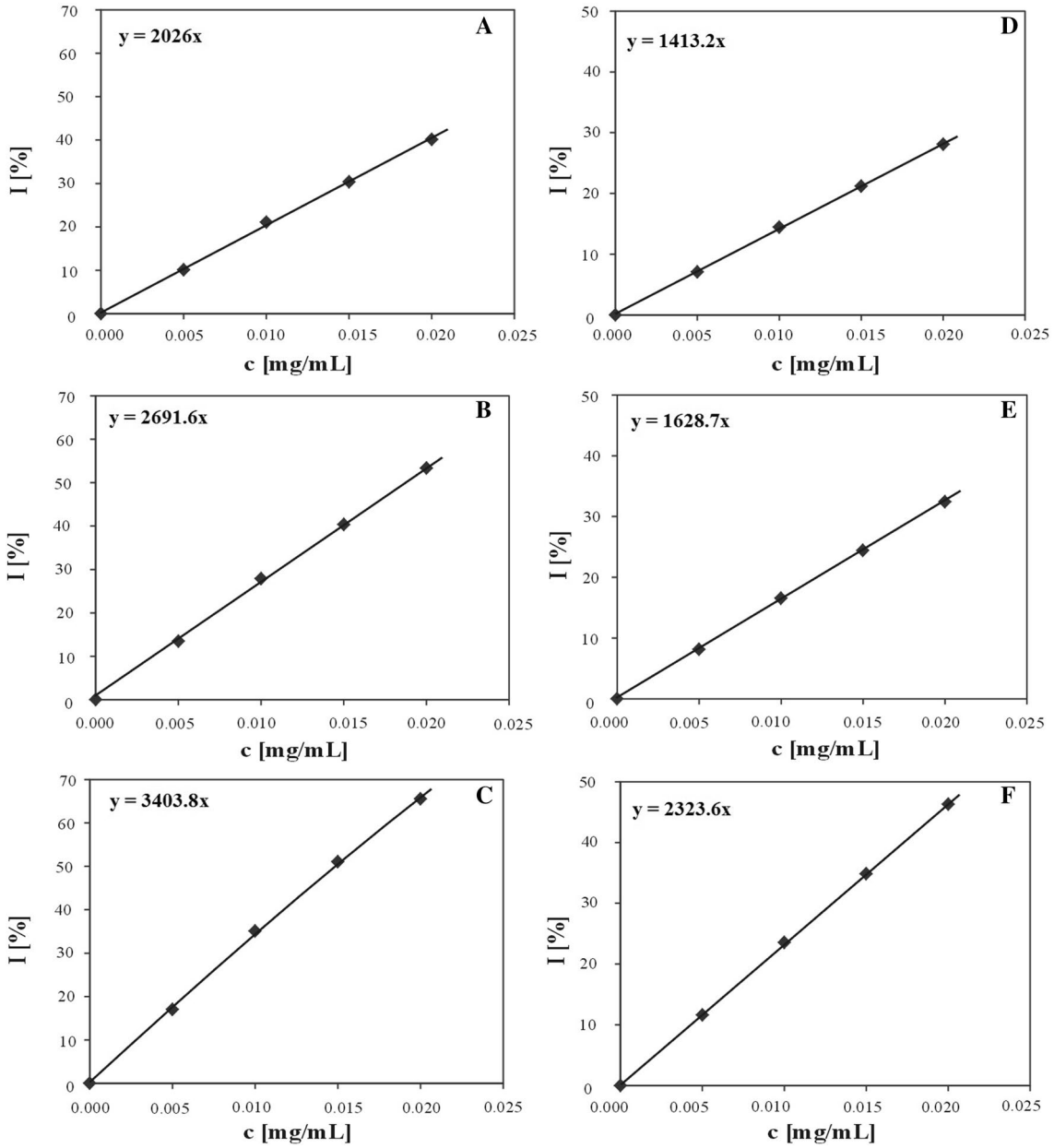


Fig. 7 The antioxidant activity changes for systems containing different concentrations of: ferulic acid solution (**a, b**), caffeic acid solution (**c, d**), gallic acid solution (**e, f**), estimated by ABTS assay in water/ethanol, 60/40 v/v at absorbance of the cation radical equal 0.5 (**a, c, e**) and 1.0 (**b, d, f**). Experimental values are mean values for $n = 3$

ABTS cation radicals, for caffeic acid (bars with dots), gallic acid (bars with chequered pattern), ferulic acid solutions (white bars) and for their binary mixtures (bars with diagonal strips) composed of equal volumes of individual component solutions. In these experiments, two ABTS concentrations were applied: Abs = 0.4 and Abs = 0.5—Fig. 5a, b, respectively. 100 µL samples were introduced to the measuring systems. Figure 5 contains also the expected antioxidant activities constructed by adding up the experimental activity data for each examined antioxidant (black bars labelled as "Σ")—e.g., bar labelled as Σ_1 represents inhibition percent which should be exhibited by the mixture containing 50 µL of caffeic acid and 50 µL of ferulic acid, assuming antioxidant effect additivity. As results from the presented data, the expected antioxidant properties of binary mixtures are greater than those experimentally determined. The observed antagonistic antioxidant effects are more evident at lower ABTS concentration (comparing individual data from Fig. 5a, b). The greatest antagonistic effect is for the binary mixture composed of

Table 3 The significance (*T* and *p* values) of the difference between the experimental and expected antioxidant activity for ternary mixture of the examined antioxidants

Sample	Solvent							
	Water		Ethanol/water (15/85 v/v)		Ethanol/water (40/60 v/v)		Ethanol	
	T	*p*	*T*	*p*	*T*	*p*	*T*	*p*
1 4 7 Σ 10	9.045	0.00004	9.894	0.00002	21.661	< 0.00001	4.925	0.00017
1 5 8 Σ 11	5.967	0.00056	2.381	0.04881	17.077	< 0.00001	6.748	0.00027
2 4 8 Σ 12	8.356	0.00007	13.649	< 0.00001	23.967	< 0.00001	5.330	0.00011
2 6 7 Σ 13	15.138	< 0.00001	14.173	< 0.00001	26.880	< 0.00001	0	1
2 5 9 Σ 14	5.809	0.00065	7.591	0.00013	18.875	< 0.00001	3.930	0.00567
3 6 8 Σ 15	9.542	0.00002	10.031	0.00002	22.539	< 0.00001	12.708	< 0.00001
3 4 9 Σ 16	7.757	0.00011	9.340	0.00003	24.142	< 0.00001	11.036	0.00001

Table 4 Depletion of ferulic acid in measuring systems containing one antioxidant and its binary mixture

	Number of mono- and di-antioxidant system—see Table 1					
	7	18	21	8	17	22
Depletion of ferulic acid (%)	98.76	96.92	98.66	97.00	96.90	97.36

The results were calculated using HPLC analysis

Table 5 Depletion of ferulic acid in measuring systems containing one antioxidant and its ternary mixture

	Number of mono- and tri-antioxidant system—see Table 2									
	7	10	13	8	11	12	15	9	14	16
Depletion of ferulic acid (%)	97.11	89.51	94.70	99.00	79.03	86.02	90.32	100	72.51	84.14

The results were calculated using HPLC analysis

Table 6 Depletion of gallic acid in measuring systems containing one antioxidant and its binary mixture

	Number of mono- and di-antioxidant system—see Table 1					
	2	17	26	3	18	25
Depletion of gallic acid (%)	100.00	99.13	99.06	100	99.19	99.40

The results were calculated using HPLC analysis

Table 7 Depletion of gallic acid in measuring systems containing one antioxidant and its ternary mixture

	Number of mono- and tri-antioxidant system—see Table 2									
	4	10	12	16	5	11	14	6	13	15
Depletion of gallic acid (%)	100	98.81	98.10	98.95	100	98.90	99.23	100	98.90	98.95

The results were calculated using HPLC analysis

Table 8 The significance (*T* and *p* values) of the difference between the experimental and expected antioxidant activity for binary mixture of the examined antioxidants

Components	*A* = 0.500		*A* = 0.400	
	T	*p*	*T*	*p*
Gallic/ferulic	17.35	6.47E−05	94.68	7.5E−08
Ferulic/caffeic	7.19	1.98E−03	13.64	1.7E−04
Caffeic/gallic	8.72	9.51E−04	32.10	5.6E−06

gallic and ferulic acids. The significance of the difference between the experimental and expected antioxidant activity of binary mixtures has been estimated by the *T* and *p* values listed in Table 8.

Figures 6 and 7 show the results of the additional experiments performed to establish the relationships between the ABTS cation radical inhibition percent and the concentration of ferulic (A and D), caffeic (B and E) and gallic acid (C and F) at two different concentrations of $ABTS^{\bullet+}$ (Abs = 0.5—see A, B and C; Abs = 1.0—see D, E and F) and at two different ethanol concentrations (15%—Fig. 6;

40%—Fig. 7). The slope of the plot of the relationships (see linear equations at individual plots) is a measure of reaction velocity between radical and antioxidant. The presented data confirm the literature reports concerning reaction kinetics, particularly its dependence on reagent concentration and solvent type, and also prove that the reaction velocity between the ABTS cation radical and gallic acid is the quickest. Hence, the data from Figs. 6 and 7 confirm the validity of the hypothesis that the differences in reactions kinetics between the given antioxidant and the ABTS cation radical is responsible for the observed antagonistic effect in ternary mixtures: the depletion degree of ABTS cation radicals in a time unit by gallic acid (the quickest antioxidant) is the greatest. In consequence, the accessibility of ABTS cation radicals for weaker antioxidants molecules (caffeic and ferulic acid) is decreased. At a higher ABTS cation radicals concentration ($A = 0.8$), the antagonistic antioxidant effect in binary mixtures is not observed (see Figs. 1, 2, 3). Despite quick neutralization of the radicals by the strongest antioxidant, their concentration is high enough to maintain the stability of the reaction kinetics with a weaker antioxidant. The addition of another competitor to the radicals at $A = 0.8$ causes their faster consumption and slows down the kinetics of their reaction with weaker antioxidants. Hence, the antagonistic antioxidant effect is observed in ternary mixtures (see Fig. 4).

The present study, like other numerous published scientific works and inquiries, attempts to explain the reasons of the antagonistic antioxidant effect observed in various mixtures of phenolic substances. The obtained data proved that the experimentally observed antioxidant antagonism in mixtures of antioxidants does not result from mutual interaction between individual antioxidants causing the change of their radical neutralization ability but is the effect of the difference in reaction kinetics between a given antioxidant and the ABTS cation radical. The magnitude of the experimentally observed antagonistic effect of antioxidant mixture depends on both the type of individual mixture components and their mutual quantitative relations. These conclusions were formed based on experiments with binary and ternary mixtures of gallic, ferulic and caffeic acids solutions of concentrations similar to those found in wine. To confirm them, further experiments with other antioxidant systems differing in qualitative and quantitative composition are required. Especially as results from literature [27, 28] show that the antioxidant interaction was affected by the ratios of phytochemicals. Detailed knowledge concerning the antagonistic effect in various antioxidant mixtures can be helpful in designing functional foods and supplements.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

Compliance with ethics requirements This article does not contain any studies with human and animals subjects.

References

1. Heo HJ, Kim YJ, Chung D, Kim DO (2007) Antioxidant capacities of individual and combined phenolics in a model system. Food Chem 104:87–92
2. Peyrat-Maillard MN, Cuvelier ME, Berset C (2003) Antioxidant activity of phenolic compounds in 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation: synergistic and antagonistic effects. J Am Oil Chem Soc 80:1007–1012
3. Hidalgo M, Sánchez-Moreno C, de Pascual-Teresa S (2010) Flavonoid-flavonoid interaction and its effect on their antioxidant activity. Food Chem 121:691–696
4. Le Grandois J, Guffond D, Hamon E, Marchioni E, Werner D (2017) Combined microplate-ABTS and HPLC-ABTS analysis of tomato and pepper extracts reveals synergetic and antagonist effects of their lipophilic antoxidative components. Food Chem 223:62–71
5. Pinelo M, Manzocco L, Nuñez MJ, Nicoli MC (2004) Interaction among phenols in food fortification: negative synergism on antioxidant capacity. J Agric Food Chem 52:1177–1180
6. Nile SH, Ko EY, Kim DH, Keum YS (2016) Screening of ferulic acid related compounds as inhibitors of xanthine oxidase and cyclooxygenase-2 with anti-inflammatory activity. Bras J Pharmacogn 26:50–55
7. Rocha LD, Monteiro MC, Teodoro AJ (2012) Anticancer properties of hydroxycinnamic acids—a review. Cancer Clin Oncol 1:109–120
8. Shalini Srivastava R (2009) Antifungal activity screening and HPLC analysis of crude extract from Tectona grandis, Shilajit, Valeriana wallachi. Electron J Environ Agric Food Chem 8:218–229
9. Kikuzaki H, Hisamoto M, Hirose K, Akiyama K, Taniguchi H (2002) Antioxidant properties of ferulic acid and its related compounds. J Agric Food Chem 202:2161–2168
10. Zhao HX, Zhang HS, Yang SF (2014) Phenolic compounds and its antioxidant activities in ethanolic extracts from seven cultivars of Chinese jujube. Food Sci Hum Wellness 3:183–190
11. Nenandis N, Wang LF, Tsimidou M, Zhang HY (2004) Estimation of scavenging activity of phenolic compounds using the ABTS assay. J Agric Food Chem 52:4669–4674
12. Dawidowicz AL, Olszowy M, Jóźwik-Dolęba M (2015) Antagonistic antioxidant effect in butylated hydroxytoluene/butylated hydroxyanisole mixture. J Food Process Preserv 39:2240–2248
13. Dawidowicz AL, Olszowy M (2013) The importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS assay. Eur Food Res Technol 236:1099–1109

14. Dawidowicz AL, Olszowy M, Jóźwik-Dolęba M (2015) Importance of solvent association in the estimation of antioxidant properties of phenolic compounds by DPPH method. J Food Sci Technol 52:4523–4529
15. Dawidowicz AL, Olszowy M (2011) Antioxidant properties of BHT estimated by ABTS assay in systems differing in pH or metal ion or water concentration. Eur Food Res Technol 232:837–840
16. Aoun M, Makris DP (2012) Binary mixtures of natural polyphenolic antioxidants with ascorbic acid: impact of interactions on the antiradical activity. Int Food Res J 19:603–606
17. Choe E, Min DB (2009) Mechanisms of antioxidants in the oxidation of foods. Compr Rev Food Sci Food Saf 8:345–358
18. Rúa J, De Arriaga D, Garcia-Armesto MR, Busto F, Del Valle P (2017) Binary combination of natural phenolic compounds with gallic acid or with its alkyl esters: an approach to understand the antioxidant interaction. Eur Food Res Technol 243:1211–1217
19. Osman AM, Wong KKY, Fernhough A (2006) ABTS radical-driven oxidation of polyphenols and structural elucidation of covalent adducts. Biochem Biophys Res Commun 346:321–329
20. Li W, Zhang Y, Liu Y, Yue F, Lu Y, Qiu H, Gao Y, Wu Y, Wang Z, Huang R, Zhang C (2012) In vitro kinetic evaluation of the free radical scavenging ability of propofol. Anesthesiology 116:1258–1266
21. Liu YR, Li WG, Chen LF, Xiao BK, Yang JY, Yang L, Zhang CG, Huang RQ, Don JX (2014) $ABTS^{+}$ scavenging potency of selected flavonols from *Hypericum perforatum* L. by HPLC-ESI/MS QQQ: reaction observation, adduct characterization and scavenging activity determination. Food Res Int 58:47–58
22. Tai A, Ohno A, Ito H (2016) Isolation and characterization of the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation—scavenging reaction product of arbutin. J Agric Food Chem 64:7285–7290
23. Rudolphi-Skórska E, Filek M, Zembala M (2015) α-Tocopherol/gallic acid cooperation in the protection of galactolipids against ozone-induced oxidation. J Membr Biol 249:87–95
24. Chen Z, Ren J, Li Y, Chang W, Chen Z (2002) Phenolic antioxidants: electrochemical behavior and the mechanistic elements underlying their anodic oxidation reaction. Redox Rep 7:395–402
25. Teixeira J, Gaspar A, Garrido EM, Garrido J, Borges F (2013) Hydroxycinnamic acid antioxidants: an electrochemical overview. BioMed Res Int 1:1–12
26. Sousa RW, Da Rocha C, Cardoso CL, Silva SDH, Zanoni BM (2004) V. Determination of the relative contribution of phenolic antioxidants in orange juice by voltammetric methods. J Food Compos Anal 17:619–633
27. Pan Z, Li H, Zheng S, Zhang B, Deng Z-Y (2018) Implication of the significance of dietary compatibility: based on the antioxidant and anti-inflammatory interactions with different ratios of hydrophilic and lipophilic antioxidants among four daily agricultural crops. J Agric Food Chem 66:7461–7474
28. Pan Z, Deng S, Chen X, Zhang B, Li H (2018) Daily dietary antioxidant interactions are due to not only the quantity but also the ratios of hydrophilic and lipophilic phytochemicals. J Agric Food Chem 66:9107–9120