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Reactions of Flavonoids with *o*-Quinones Interfere with the Spectrophotometric Assay of Tyrosinase Activity

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Supporting Information

ABSTRACT: Flavonoids are important food components with antioxidant properties and many of them have been described as tyrosinase inhibitors. Oxidation of quercetin, kaempferol, morin, catechin, and naringenin by mushroom tyrosinase and their influence on the oxidation of L-dopa and L-tyrosine was studied. Reaction rates measured spectrophotometrically and by oxygen consumption differed substantially. All tested flavonoids reacted with 4-*tert*-butyl-*o*-benzoquinone and/or 4-methyl-*o*-benzoquinone, although at different rates. These reactions generated products whose UV−vis spectra either overlapped or did not overlap with the spectrum of dopachrome. They therefore strongly influence the kinetic analysis performed by measuring the absorbance at 475 nm during oxidation of L-dopa or L-tyrosine generating false inhibition or activation effects. This method is therefore inappropriate for monitoring the activity of this enzyme in the presence of flavonoids and other compounds possessing strong nucleophilic or reducing groups.

KEYWORDS: *tyrosinase, flavonoids, o-quinone, catechol, redox exchange, enzymatic assay interference*

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a key enzyme in the melanogenesis pathway catalyzing the two initial and rate limiting steps: hydroxylation of L-tyrosine to L-dopa and its subsequent oxidation to dopaquinone, which then undergoes a series of nonenzymatic and enzymatic reactions leading to melanins.[1] Its excessive activity is involved in several pigmentation disorders and darkening of many food products. Therefore, the search for inhibitors of this enzyme is quite extensive and a large number of such compounds have been isolated from natural sources.[2]

Flavonoids constitute a large group of natural products with antioxidant properties[3] and a number of them have been described as tyrosinase inhibitors.[4] These studies were initiated in the 1990s by the work of Kubo and co-workers with the discovery that flavonoids isolated from American plants strongly inhibit this enzyme.[5,6] Quercetin and kaempferol were particularly well-studied.[7,8] For quercetin a detailed kinetic analysis of this inhibition was performed.[9] Later, however, it was demonstrated that this compound also served as a substrate for tyrosinase.[10,11] Subsequently, it was shown that it was oxidized to the corresponding *o*-quinone, which isomerized to the *p*-quinone methide and this intermediate underwent addition of water molecule to yield a relatively stable protocatechuate derivative—2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone.[12] Recently, the number of articles describing flavonoids which inhibit tyrosinase has been growing rapidly. Such an activity has been reported for morin,[13,14] fisetin and galangin,[13] isorhamnetin,[15] luteolin,[16] uralenol and broussoflavonol F,[17] pedalitin,[18] and genkwain,[19] to name just a few.

However, since flavonoids are polyphenols and many of them contain a catechol group, they may also be oxidized by tyrosinase, as it has been already demonstrated for quercetin[10−12] and morin.[11] They are also known antioxidants and this property is determined in standard redox reactions, usually with stable radicals, such as $DPPH^{\bullet}$ and $ABTS^{+\bullet}$, frequently at the same time as their tyrosinase inhibitory activity is studied.[20−22] Since these compounds readily reduce free radicals, they may therefore also undergo redox reactions with *o*-quinones generated by tyrosinase. We have therefore studied the oxidation of common flavonoids, such as quercetin, kaempferol, morin, catechin, and naringenin, by tyrosinase and their influence on the oxidation of L-dopa and L-tyrosine by spectrophotometric and oxymetric techniques. Here we demonstrate that these compounds react with *o*-quinones and these reactions strongly influence the results of spectrophotometric analysis of reactions catalyzed by this enzyme.

MATERIALS AND METHODS

Chemicals and Reagents. L-Tyrosine, L-dopa, quercetin, kaempferol, morin, catechin, naringenin, 4-*tert*-butylcatechol, and 4-methylcatechol were purchased from Sigma-Aldrich (Poznań, Poland). Sodium periodate, solvents, and buffer components were from Avantor Performance Materials (Gliwice, Poland). Tyrosinase was purified from mushrooms (*Agaricus bisporus*) as described before.[23]

Spectrophotometric Measurements. Due to the low solubility of flavonoids their 1 mM solutions were prepared in water/DMSO mixtures containing: 30% DMSO for quercetin and kaempferol, 10% DMSO for morin, and 5% DMSO for catechin and naringenin. The final DMSO concentration in reaction mixtures ranged therefore from 0.05 to 3%. Reactions were carried out in 3 mL of 50 mM sodium phosphate buffer, pH 6.8 at room temperature. Oxidation of flavonoids by tyrosinase was carried out with 50 or 100 μM substrate and 15, 30, or 60 μg of tyrosinase. Spectra from 200 to 700 nm were recorded in a Jasco V-650 UV−vis spectrophotometer immediately after mixing the reagents and then in 1 min intervals for 30−60 min. Reactions of mixtures of flavonoids with L-dopa or L-tyrosine were carried out at 100 μM L-tyrosine or L-dopa and 10, 25, 50, or 100 μM flavonoids and

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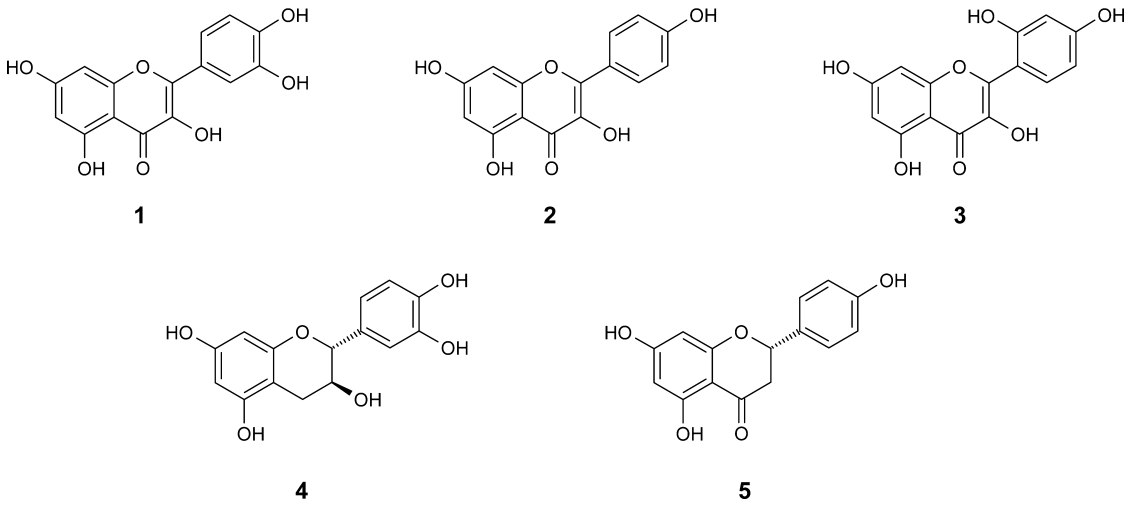

Figure 1. Structures of flavonoids used in this study: **1**: quercetin; **2**: kaempferol; **3**: morin; **4**: catechin; and **5**: naringenin.

the spectra were recorded as for the flavonoids alone. Additionally the same reactions were performed and the absorbance at 475 nm was measured for 30 min. For reactions of flavonoids with 4-*tert*-butyl-*o*-benzoquinone and 4-methyl-*o*-benzoquinone the *o*-quinones were generated by oxidation of the corresponding catechols (4-*tert*-butylcatechol and 4-methylcatechol) at 75 μM with 50 μM sodium periodate (50% excess of catechols was used to ensure consumption of periodate). The reaction mixtures were incubated for 5 min and their spectra were taken at 1 min intervals. In a parallel reaction the same reagents were mixed in a smaller volume, which would allow reaching the final concentration after addition of flavonoids. The flavonoids were then added to reach a final concentration of 50 μM and the spectra were recorded for 30−60 at 1 min intervals.

Oxygen Consumption Measurements. Measurements were performed with a multifunctional electrochemical device CX-551 (ELMETRON, Zabrze, Poland) equipped with an oxygen (Clark-type) sensor CTN-9506 (ELSENT, Wrocław, Poland) connected to a computer. The sensor was calibrated according to manufacturer's instruction using a two-point method with saturated sodium sulfite solution (0% point) and air-saturated distilled water (100% point). Reactions were carried out in 15.0 mL of 50 mM sodium phosphate buffer, pH 6.8 under conditions identical to spectrophotometric measurements (with the amount of the enzyme scaled up appropriately).

RESULTS

We have selected for our analysis five flavonoids (quercetin, kaempferol, morin, catechin, and naringenin, Figure 1), which have been described as substrates,[11,12,24] inhibitors,[7,8,13,14] and/or activators[19,25] of tyrosinase. The first three compounds (quercetin, kaempferol, morin) contain phenolic groups in ring B conjugated with the 3-OH group in ring C. They can therefore, after oxidation, tautomerize to the corresponding *p*-quinone methides and form the protocatechuate products, which have been obtained after oxidation of quercetin by tyrosinase[12] and all three compounds by Cu^{2+} ions.[26] Quercetin contains a catechol group in ring B and can therefore serve as an *o*-diphenolic substrate of tyrosinase and can be oxidized by both the *oxy* and *met* forms of this enzyme. Kaempferol contains a monophenol in ring B, whereas morin contains a *m*-diphenol in ring B—they can therefore only act as monophenolic substrates of tyrosinase. However, in both compounds the phenolic groups in ring B (4′−OH in kaempferol and morin, as well as 2′−OH in morin) are conjugated with the 3-OH group. Catechin and naringenin contain phenolic groups in ring B but they are either not conjugated to the 3-OH group in ring C (catechin) or the 3-OH group is not present (naringenin). They are therefore incapable of forming *p*-quinone methides and their oxidation should be limited to ring B.

We began our analysis by recording the UV−vis spectra of the mixtures of the selected flavonoids with tyrosinase to be able to compare them with the spectra of reaction mixtures containing L-dopa or L-tyrosine. A reference reaction with L-dopa was also carried out (Figure 2A). The reactivity of the enzyme toward each compound differed substantially. The spectra recorded during oxidation of quercetin were essentially identical to those reported previously,[10,12] with a new maximum appearing at 335 nm (Figure 2B), indicating the formation of the protocatechuate product—2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2*H*)-benzofuranone.[12] When kaempferol was subjected to this reaction, only a shoulder at ca. 450 nm appeared (Figure 2C). Very little changes in the UV−vis spectrum were also observed during incubation of tyrosinase with morin (Figure 2D), which was previously reported as a substrate of tyrosinase.[11] However, when kaempferol was incubated with larger amounts of tyrosinase (up to 600 μg), its oxidation became obvious. These reactions generated a broad absorption between 250 and 500 nm indicating formation of a complex mixture of products (Figure S1). This is most likely due to the low rate of enzymatic oxidation and complexity of reactions occurring in the mixture (enzymatic formation of the *o*-quinone in ring B, its subsequent tautomerization to the quinone methide analogous to the reaction described for quercetin,[12] redox and nucleophilic addition reactions of this *o*-quinone with the substrate, see below). Catechin was rapidly oxidized as described before[24] generating products (most likely adducts and polymers) with a broad spectrum ranging from 300 to 600 nm (Figure 2E), resembling the spectrum observed also during oxidation of eriodictyol.[27] Oxidation of naringenin generated a broad absorbance between 350 and 550 nm (Figure 2F), similar to the spectrum obtained with catechin, although the rate of this reaction was much smaller. This would be expected, however, since naringenin contains only a phenol group in ring B, which would have to be hydroxylated first in a monooxygenase reaction analogous to tyrosine. The low rates of oxidation of kaempferol, morin, and naringenin can be explained by their inability to recruit the *met* form of tyrosinase to the catalytic cycle.

It should be pointed out that the spectra of some of these products overlap with the spectrum of dopachrome and would

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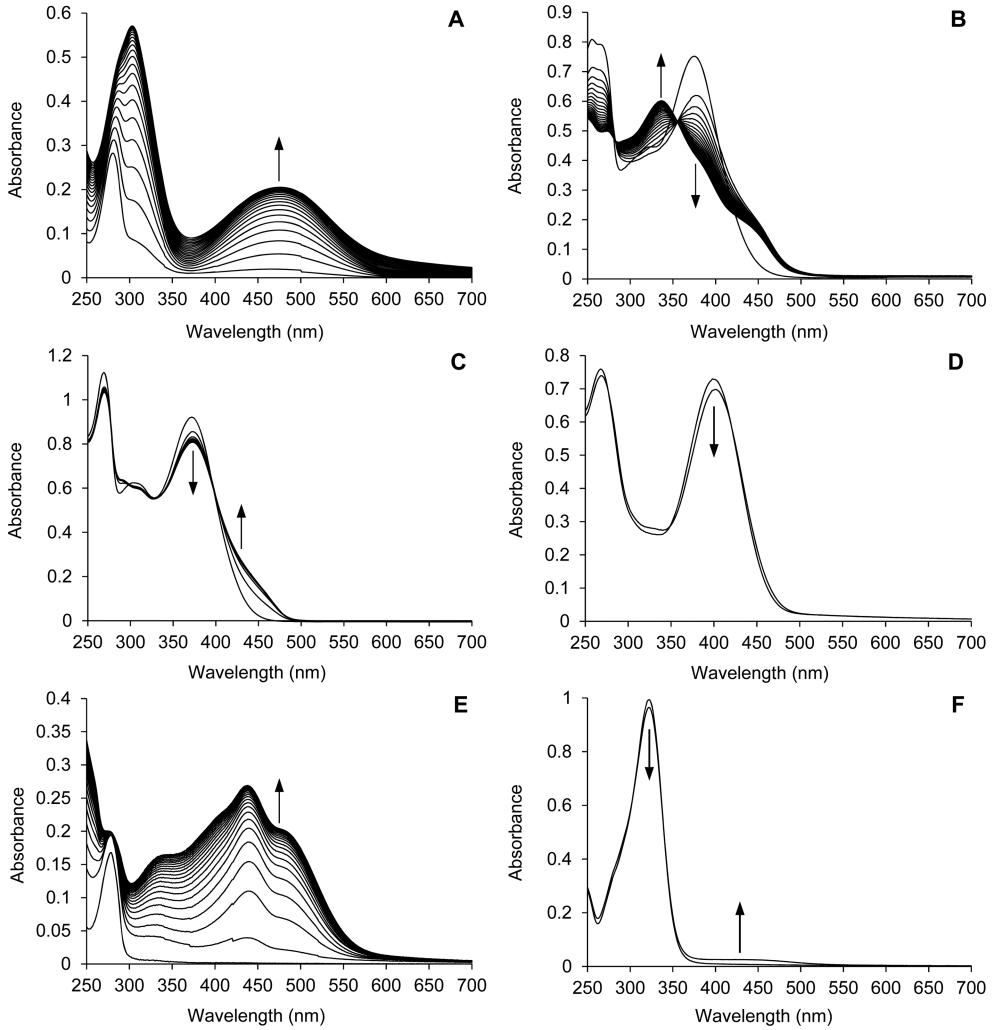

Figure 2. UV−vis spectra recorded during oxidation of 100 μM L-dopa (A), 50 μM quercetin (B), 50 μM kaempferol (C), 50 μM morin (D), 50 μM catechin (E), and 50 μM naringenin (F) by tyrosinase (60 μg of the enzyme was used for morin, which reacted most slowly, whereas 15 μg was used for all other compounds). Spectra were recorded in 1 min intervals for 20 min. For morin (D) and naringenin (F) only the initial and final spectra were displayed.

therefore interfere with the kinetic analysis performed by monitoring tyrosinase activity spectrophotometrically at 475 nm. This effect became clear when mixtures of the flavonoids with L-dopa or L-tyrosine were oxidized by tyrosinase (Figure 3). The spectra of reaction mixtures containing quercetin at higher concentration (50 and 100 μM) indicated that in the initial phase of the reaction only the oxidation product of this flavonoid was formed (Figure 3A and F), as it has been described before.[12] Almost no absorbance in the visible region of dopachrome absorption was detected. For other flavonoids it was not possible to determine from the UV−vis spectra which compounds were oxidized and what products were formed, although the rates of changes in the spectra in the dopachrome absorption region differed substantially (Figure 3B−E and G−J).

When these reactions were monitored by measuring the absorbance at 475 nm, it became obvious that if the kinetic analysis were performed by single-time-point measurements, the results would strongly depend on the time of measurements. During oxidation of L-dopa changes in absorbance were slightly bigger in the initial phase of the reaction in the presence of quercetin, kaempferol, and morin at lower concentrations (10 and 25 μM) (Figure 4A−C), which may be attributed to a small absorption of their oxidation products in this region (Figure 2B, C, and D). Later, however, kaempferol in the whole concentration range and quercetin at 25, 50, and 100 μM decreased the changes in absorbance in a concentration-dependent manner (Figure 4A,B). After the initial phase morin had no effect on the changes in absorbance at lower concentrations. Only at 100 μM moderate decrease in absorbance was observed (Figure 4C). In the presence of catechin the changes in absorbance were much bigger than without this compound (Figure 4D), which is due to the overlap of the spectrum of the oxidation products of this flavonoid (Figure 2E) and dopachrome (Figure 2A). Naringenin slightly decreased the changes in absorbance only at higher concentrations (50 and 100 μM, Figure 4E).

Different effects, however, were observed during oxidation of L-tyrosine. It should be pointed out that all the tested compounds, with the exception of naringenin, which does not possess a catechol group or conjugated phenolic groups in rings B and C, reduced or eliminated the lag phase in the monophenolase reaction in spectrophotometric measurements. So, in fact changes of absorbance in the initial phase of the reactions were bigger in the presence of quercetin, kaempferol, morin, and catechin at most concentrations than in their absence (Figure 4F−I).

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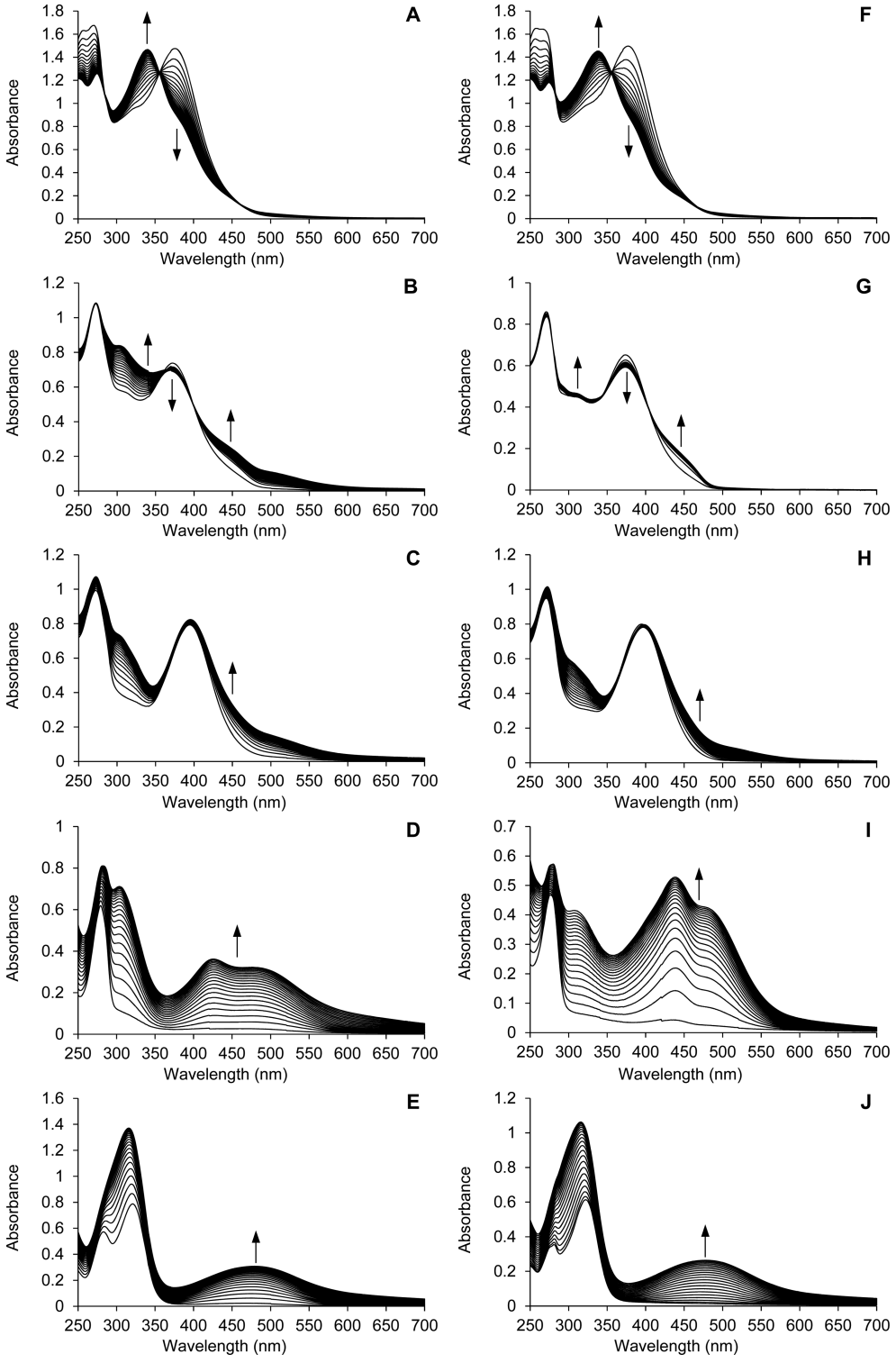

Figure 3. UV−vis spectra recorded during oxidation of mixtures of 100 μM L-dopa with 100 μM quercetin (A), 50 μM kaempferol (B), 50 μM morin (C), 100 μM catechin (D), and 25 μM naringenin (E) and mixtures of 100 μM L-tyrosine with 100 μM quercetin (F), 50 μM kaempferol (G), 50 μM morin (H), 100 μM catechin (I), and 25 μM naringenin (J) by tyrosinase (15 μg of the enzyme was used in reactions with L-dopa and 30 μg in reactions with L-tyrosine). The concentrations of flavonoids were selected to best show the changes in the spectra in comparison with L-dopa or L-tyrosine alone. Spectra were recorded in 1 min intervals for 20 min.

Later, however, quercetin, kaempferol, and morin significantly reduced the changes of absorbance, while naringenin had little effect on this parameter in the whole measurement period (Figure 4J).

When oxygen consumption was monitored, however, the picture was quite different. Quercetin, kaempferol, and morin decreased the rate of the diphenolase reaction in a concentration-dependent manner (Figure 5A−C), catechin increased it (Figure 5D), while the effect of naringenin was negligible (Figure 5E). In the monophenolase reaction quercetin, morin, and catechin reduced or abolished the lag phase. Later, however, both quercetin and morin strongly reduced the reaction rate (Figure 5F,H), while

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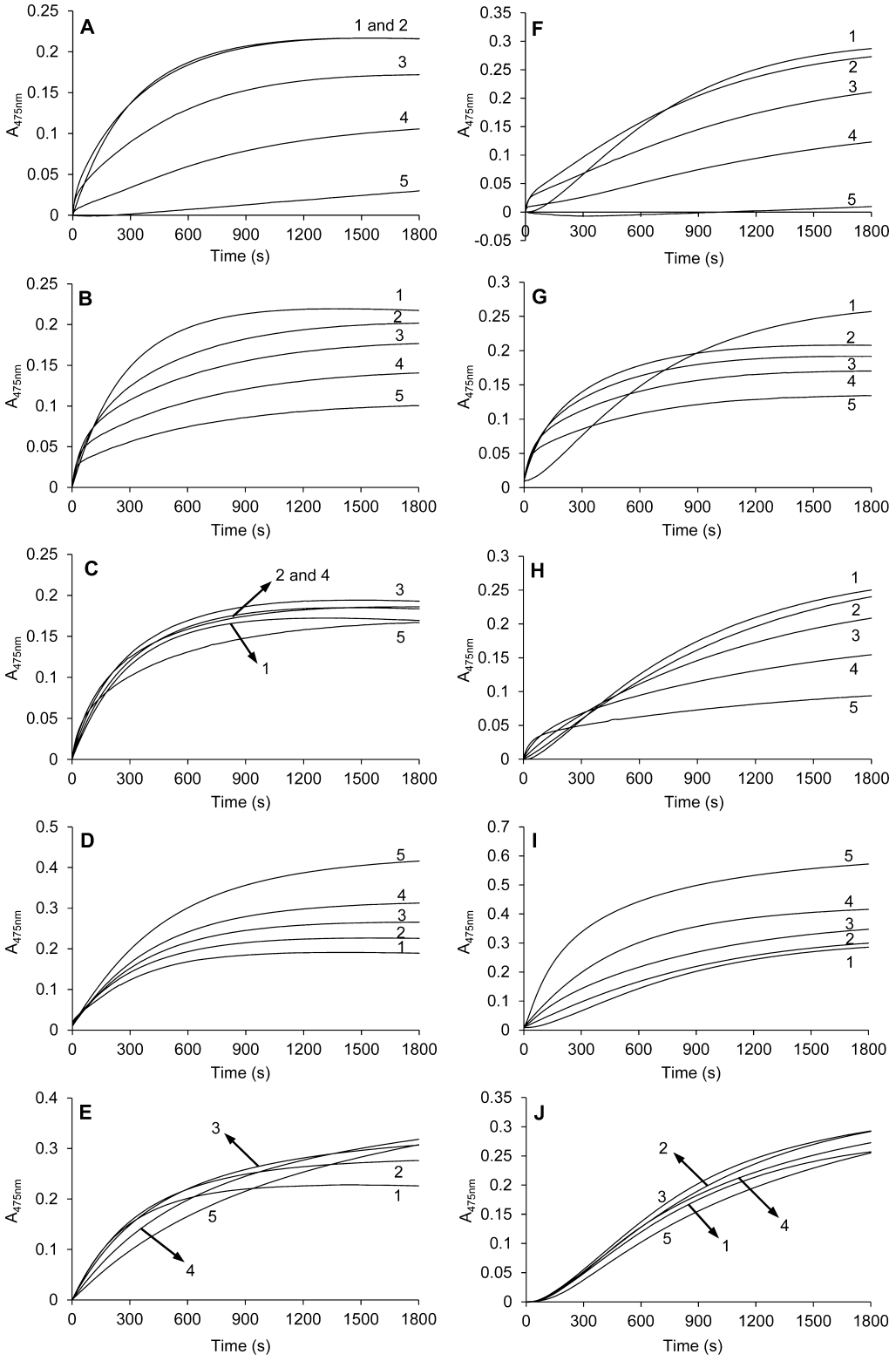

Figure 4. Absorbance at 475 nm measured during oxidation of 100 μM L-dopa (A–E) or 100 μM L-tyrosine (F–J) with quercetin (A and F), kaempferol (B and G), morin (C and H), catechin (D and I), and naringenin (E and J) by tyrosinase (15 μg of the enzyme was used in reactions with L-dopa and 30 μg in reactions with L-tyrosine). Reactions were carried out in the absence of flavonoids (1) or their presence at 10 μM (2), 25 μM (3), 50 μM (4), or 100 μM (5) concentration.

catechin increased it (Figure 5I). Kaempferol strongly inhibited the reaction at all concentrations tested, essentially completely blocking oxygen consumption at 100 μM (Figure 5G). Naringenin reduced the reaction rate slightly only at higher concentrations (Figure 5J).

These results show that the reaction rates determined from spectrophotometric analysis and oxygen consumption measurements differ substantially for most of the tested flavonoids. If the rate of the diphenolase reaction is determined by measuring the absorbance at 475 nm, quercetin appears as the strongest

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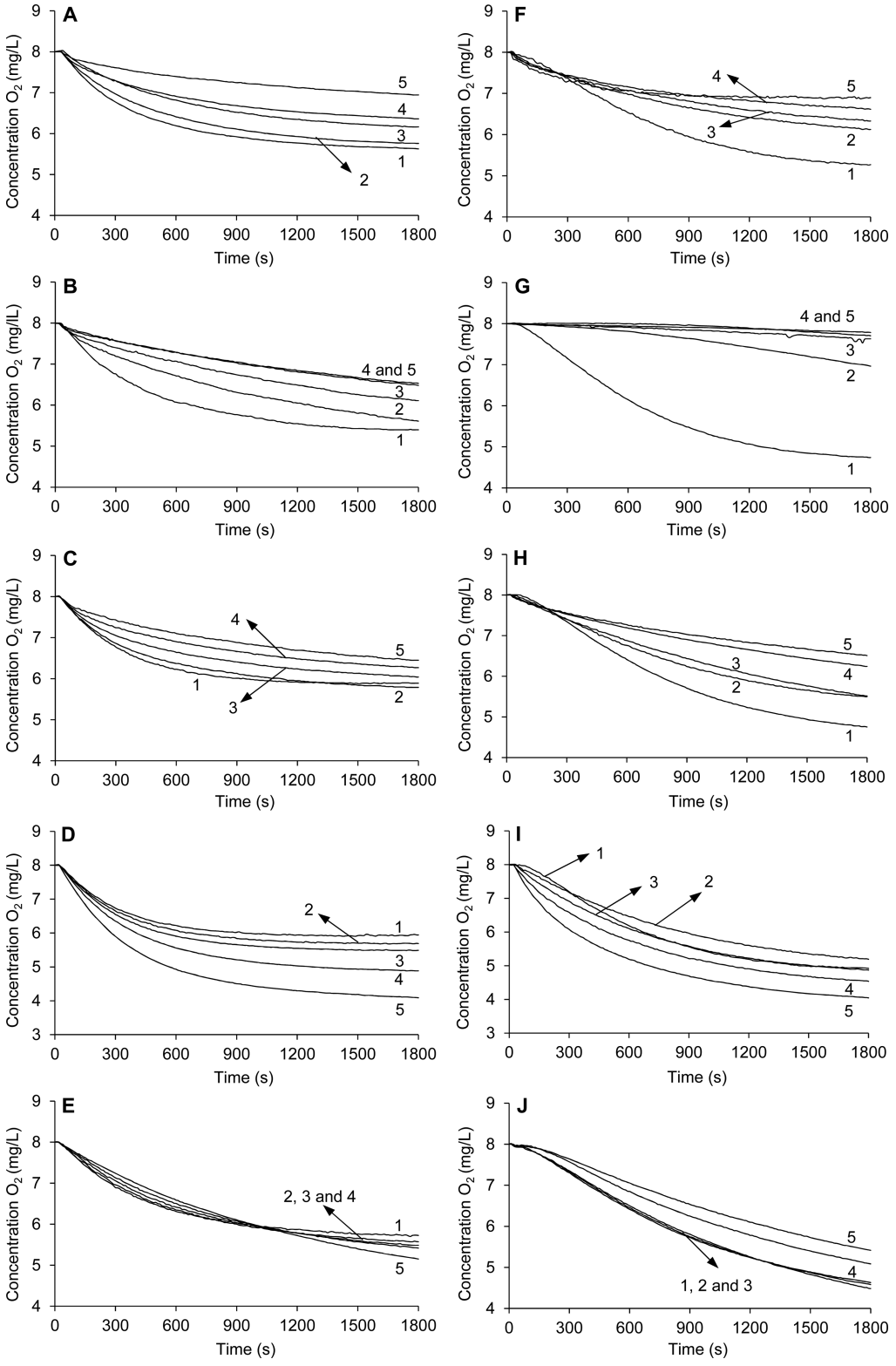

Figure 5. Oxygen consumption measurements during oxidation of 100 μM L-dopa or 100 μM L-tyrosine. Reaction conditions and labeling are the same as in Figure 4. The amount of the enzyme was scaled up appropriately to 75 and 150 μg.

inhibitor, the effect of kaempferol, morin and naringenin is modest or negligible, depending on the time of measurement, while catechin will appear as an activator. When the monophenolase activity is monitored by the same method, the results will even more strongly depend on the time of measurement. If the measurement is made during the lag phase, strong inhibition will only be observed for quercetin at higher concentrations, little inhibition will be observed for morin, while no inhibition of even enhancement of the reaction rate will be detected for quercetin at lower concentrations and kaempferol, morin, and catechin in the entire concentration range tested in our experiments. The actual reaction rates determined from oxygen consumption measurements are quite different: kaempferol is the strongest inhibitor of both the diphenolase and monophenolase activity, quercetin and

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morin have lower but comparable inhibitory potency, while the activity of naringenin is negligible. Catechin is not an inhibitor but a good substrate being oxidized at a rate comparable to L-dopa.

These discrepancies between the spectrophotometric and oxymetric measurements may be explained by several factors. Essentially all these flavonoids are oxidized by tyrosinase but generate products with different spectra—for those whose oxidation is limited to ring B (catechin, naringenin) the spectra overlap with the spectrum of dopachrome and for those whose original oxidation products tautomerize to *p*-quinone metides (quercetin, kaempferol, morin) they do not overlap. However, changes in absorbance may result not only from direct enzymatic oxidation but may be generated by additional reactions occurring in solution. Quercetin, kaempferol, morin, and catechin possess either a catechol moiety or conjugated phenolic groups in rings B and C, which can undergo a two-electron redox reaction with *o*-quinones produced by tyrosinase (e.g., dopaquinone). Phenolic groups may also form conjugates with *o*-quinones in nucleophilic addition reactions postulated already for quercetin.[28] Such reactions, however, are difficult to demonstrate with unstable *o*-quinones, such as dopaquinone, which rapidly undergoes an intramolecular Michael addition, due to the presence of an amino group in the side chain. We have therefore performed such reactions with 4-*tert*-butyl-*o*-benzoquinone and 4-methyl-*o*-benzoquinone generated chemically by oxidation of 4-*tert*-butylcatechol and 4-methylcatechol with sodium periodate. Results of these experiments confirmed that redox exchange and/or nucleophilic addition reactions of these flavonoids with *o*-quinones indeed occur (Figure 6), although their rates differ (Figure S2). 4-*tert*-Butyl-*o*-benzoquinone is resistant to nucleophilic attacks[29] and should therefore only participate in redox exchange reactions, while 4-methyl-*o*-benzoquinone may generate products of both reactions. When quercetin, kaempferol, and morin were incubated with 4-*tert*-butyl-*o*-benzoquinone, products with spectra very similar to that obtained when quercetin was oxidized by tyrosinase, with a maximum at 335 nm, were formed (Figure 6A–C). Catechin produced a product with an absorption maximum at 479 nm, essentially completely overlapping with the spectrum of dopachrome in the visible region (Figure 6D). Since the reaction proceeded slowly (Figure S2D), after 30 min unreacted 4-*tert*-butyl-*o*-benzoquinone gives a shoulder at 400 nm in this spectrum. Naringenin did not react with this compound (Figure 6E). Spectra obtained in reactions of 4-methyl-*o*-benzoquinone with quercetin, kaempferol, and morin closely resembled those obtained in reactions with 4-*tert*-butyl-*o*-benzoquinone, but additional absorptions with maxima at ca. 455 nm were detected, which indicated formation of additional products, most likely obtained in nucleophilic addition reactions (Figure 6F–H). A similar product indicated by a new maximum in the UV–vis spectrum at 440 nm was also formed in the case of naringenin (Figure 6J). Catechin generated a spectrum resembling that obtained with 4-*tert*-butyl-*o*-benzoquinone although with a much bigger intensity (Figure 6I) due to a much bigger reaction rate than with 4-*tert*-butyl-*o*-benzoquinone (Figure S2I). Although formation of a mixture of products cannot be excluded based on UV–vis spectra alone, it appears that the redox exchange reaction is preferred in the case of this flavonoid. Nevertheless, the spectra overlap with the spectrum of dopachrome and the absorption coefficient of this/these product(s) is/are much bigger than for dopachrome (at least 12 000 $M^{-1} \times cm^{-1}$). These additional bands at ca. 450 nm range cannot be attributed to decomposition products of 4-methyl-*o*-benzoquinone, which also absorb in this region (Figure S3), because the intensity of this absorption obtained in the presence of flavonoids was much bigger (5–30 times, Figure 6) than in their absence (Figure S3).

DISCUSSION

Our results can explain several observations made previously during reactions catalyzed by tyrosinase in the presence of flavonoids.

Inhibition of tyrosinase by flavonoids usually appears to be competitive, therefore these compounds have been frequently reported as chelators of copper ions in the enzyme's active site.[7,8,22,30,31] This conclusion was originally based on the UV–vis absorption spectra observed when flavonoids were mixed with tyrosinase and their comparison with the spectra of these compounds in the presence of Cu^{2+} ions.[7,8] Various structural elements have been mentioned as required for such complex formation. When kaempferol isolated from saffron flowers was identified as a tyrosinase inhibitor[7] it was also demonstrated that its 3-*O*-glucoside did not show inhibitory activity. The authors therefore concluded that the 3-OH group was necessary to chelate the copper ions in the active side of tyrosinase.[7] However, it has been shown that incubation of flavonol with Cu^{2+}, in contrast to Fe^{3+}, does not lead to changes in the UV–vis spectrum, which indicates that no complexation of Cu^{2+} ions by the 3-hydroxy-4-keto moiety occurs.[26] Also, the spectra of mixtures of kaempferol with copper sulfate and tyrosinase were quite different. Whereas the spectrum with Cu^{2+} ions showed maxima at ca. 340 and 404 nm, the spectrum with tyrosinase showed only a maximum at ca. 340 nm.[7] This discrepancy was explained by the authors by distortion of the kaempferol structure from planarity in the tyrosinase's active site.[7] Similar changes were observed when Cu^{2+} ions were added to quercetin—a bathochromic shift from 368 to 430 nm occurred.[8] However, for quercetin-3-*O*-glucoside a shift from 352 to 382 nm was also observed.[8] To explain the fact that a complex is formed even when the 3-OH group is blocked, the authors postulated that in this case the 3′,4′-diphenol moiety participated in its formation.[8] They did not explain, however, why complexation of copper by these groups was not considered in the case of unmodified quercetin.[8] Large differences in the UV–vis absorption spectra between mixtures of a flavonoid containing a catechol group (2-(3,4-dihydroxyphenyl)-7,8-dihydroxy-4H-1-benzopyran-4-one) and a flavonoid with a monophenol group (2-(4-hydroxyphenyl)-7-hydroxy-4H-1-benzopyran-4-one) with $CuSO_4$ were also interpreted as complex formation in the former case and not the latter to explain the differences in their inhibitory potency on tyrosinase.[30]

It should be pointed out, however, that Cu^{2+} ions are commonly used to oxidize catechols and such a reaction actually also occurs in the tyrosinase catalytic cycle, when *met*-tyrosinase (Cu^{2+}-Cu^{2+}) oxidizes catechols to *o*-quinones.[32] It has also been demonstrated that oxidation of flavonols possessing a 4′–OH group with Cu^{2+} (as well as with Fe^{3+}) leads to formation of protocatechuate products analogous to that obtained when quercetin is oxidized by tyrosinase. Such a reaction occurred not only for flavonols with a catechol group (3′–OH, 4′–OH), such as quercetin and fisetin, or a triol moiety (3′–OH, 4′–OH, 5′–OH), such as myricetin, but also for those containing only a single phenolic group at position 4′, such as kaempferol, or two phenolic groups not forming a catechol (2′–OH, 4′–OH), such as morin.[26,33] These results show that it is the conjugated system of the 3,4′-diphenol that undergoes oxidation. We have now

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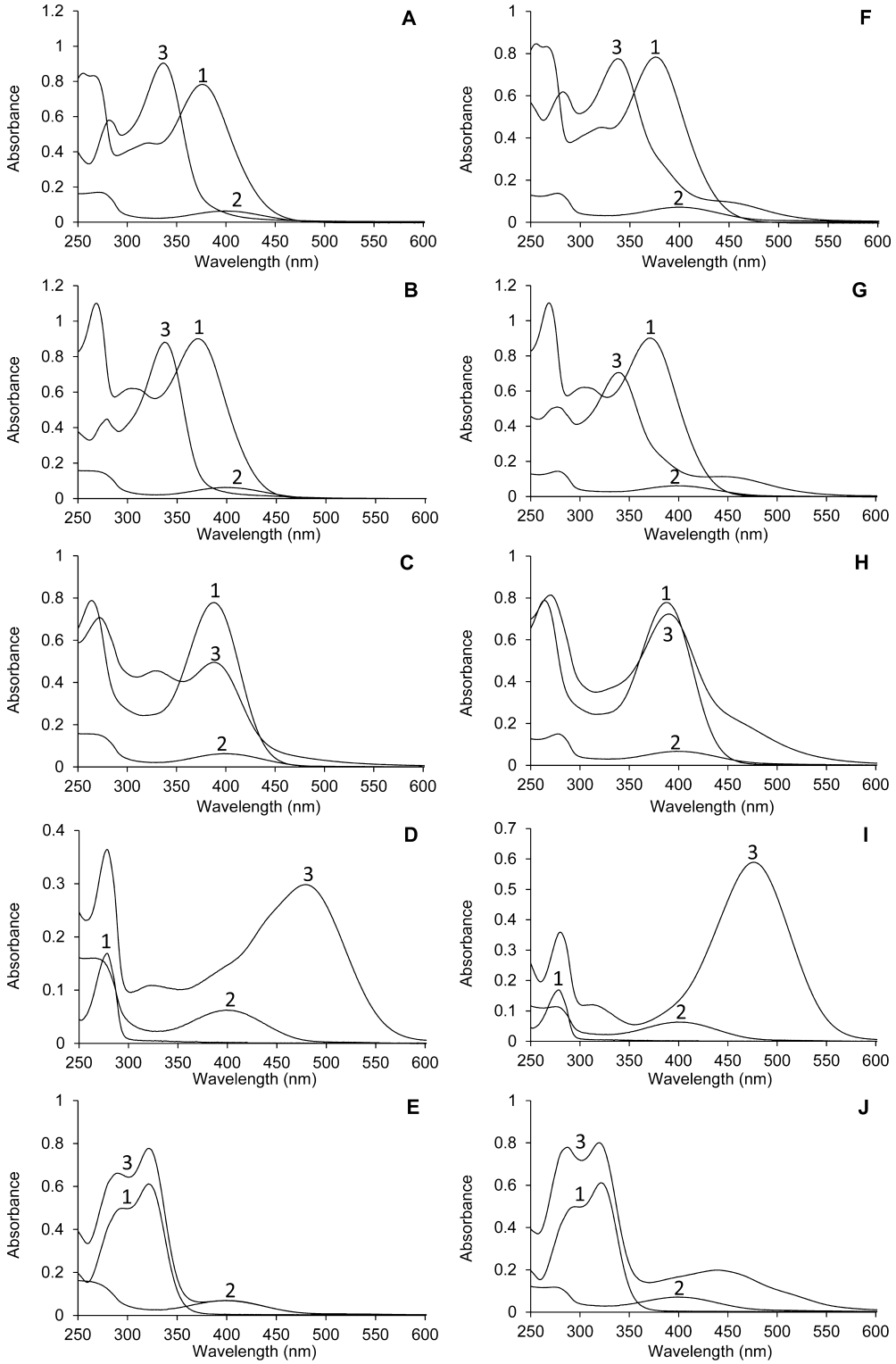

Figure 6. UV−vis spectra of the tested flavonoids (1), 4-*tert*-butyl-*o*-benzoquinone or 4-methyl-*o*-benzoquinone (2) and their mixtures after 30 min incubation (3). 4-*tert*-Butyl-*o*-benzoquinone (A−E) and 4-methyl-*o*-benzoquinone (F−J) were generated by mixing 75 μM 4-*tert*-butylcatechol or 4-methylcatechol with 50 μM sodium periodate. After 5 min the tested flavonoids at 50 μM concentration were added and the spectra were recorded for 30 at 1 min intervals: (A, F): quercetin; (B, G): kaempferol; (C, H): morin; (D, I): catechin; and (E, J): naringenin. For clarity only the final spectra of the reaction mixtures (3) are presented. All spectra showing the differences in reaction rates are presented in Figure S2.

shown that such oxidation also occurs with *o*-quinones (Figure 6). The protocatechuate products obtained in these reactions show a maximum of absorption at 330−340 nm and very low absorption at 475 nm, the wavelength used for monitoring tyrosinase activity in reactions with L-dopa or L-tyrosine. So, if the reaction involves dopaquinone, it will result in decrease of absorbance at this region and an inhibitory effect will be observed (Figure 7). This effect will depend, however, on the reaction rate between a particular flavonoid and *o*-quinone. Quercetin (and most likely fisetin), which reacts fast (Figure S2A and F), will completely prevent

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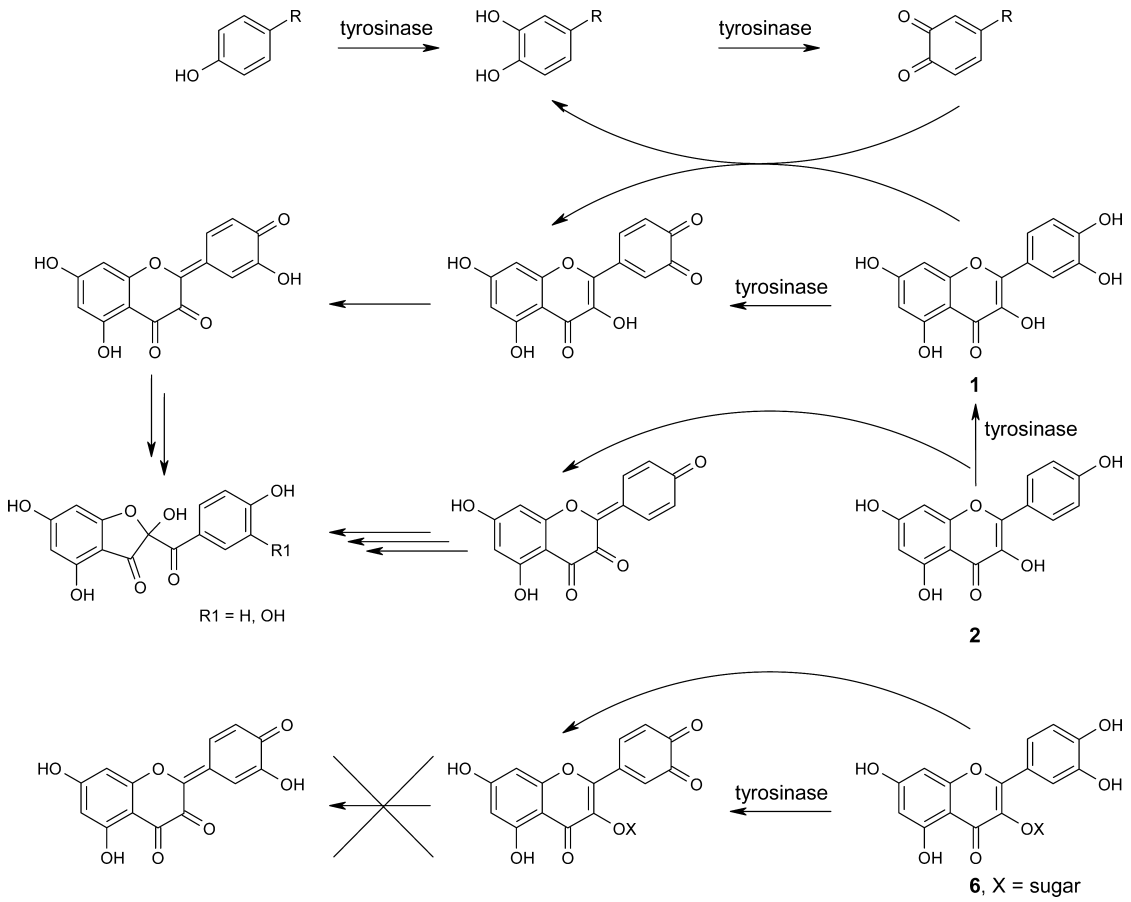

Figure 7. Comparison of the postulated reactions occurring during oxidation of quercetin (**1**), kaempferol (**2**), and quercetin 3-glycosides (**6**) by tyrosinase and *o*-quinones.

dopachrome formation (Figure 3A and F), and therefore the strongest inhibitory effect will be observed for this compound. Kaempferol and morin, on the other hand, react more slowly (Figure S2B, C, G, and H), and will therefore appear as weaker inhibitors. If, however, the 3-OH group is blocked (e.g., by alkylation or glycosylation), formation of the protocatechuate product is not possible. In the case of compounds without the catechol group (kaempferol and morin) no oxidation will occur, whereas for compounds with a catechol group (quercetin, fisetin, myricetin, rhamnetin) oxidation will lead to *o*-quinones but their tautomerization to *p*-quinone methides and subsequent transformations to the protocatechuate products will not be possible. In the latter case products with a broad absorption spectrum overlapping with the spectrum of dopachrome will be formed, as in the case of catechin (Figure 3D and I) or eriodictyol,[27] and little change or even increase of absorbance at 475 nm will be observed. Such results may then be interpreted as lack of inhibition or even stimulation of the enzyme, as it has been reported for quercetin-3-*O*-glucoside and rutin.[13] Similar results were also obtained for flavonoids isolated from the persimmon *Diospyros kaki*, where only quercetin and kaempferol showed strong inhibition of tyrosinase, while quercetin 3-glycosides isoquercitrin and hyperoside did not.[34] It is worth noting that in the same report cyanidin was shown to have inhibitory potency very similar to quercetin, whereas its 3-*O*-*β*-D-glucoside chrysontemin did not show such an activity. The structure of this antocyanidin suggest that the oxidation of its catechol group may be followed by transformations similar to the pathway demonstrated for quercetin.[12] This study, however, did not take into account the absorption of cyanidin and chrysontemin, which in large part overlap with the spectrum of dopachrome. Their oxidation will therefore inevitably have strong and complex influence on absorption at 475 nm. Our conclusions also seem to be supported by the fact that quercetin derivatives modified at the 3′-position, such as isoramnetin[15] and quercetin-3′-*O*-*β*-D-glucoside,[35] retain their tyrosinase inhibitory activity determined spectrophotometrically, in contrast to quercetin blocked at position 3.

Redox reactions with *o*-quinones may occur not only for the flavonoids which we have studied here, but also for oligomers and polymers containing flavan-3-ol subunits, such as ephedrannins,[36] proanthocyanidins,[31,37,38] and tannins,[39] as well as other polyphenols capable of undergoing a two-electron oxidation described as tyrosinase inhibitors, such as rosmarinic acid and its derivatives.[18] Nucleophilic addition and redox reactions with *o*-quinones have already been postulated for condensed tannins,[39] because their presence decreased the formation of dopachrome, when dopa was oxidized by sodium periodate in their presence. However, in that case the observed effect should be attributed to competing oxidation of tannins by this oxidant, which was added to their mixtures with L-dopa.

It was also described before that quercetin abolished the lag phase in the oxidation of L-tyrosine by tyrosinase but kaempferol did not.[7,8] For quercetin this effect may be explained by its acting as a catecholic substrate recruiting the *met*-form of the enzyme to the catalytic cycle. In our experiments, however, morin also reduced or eliminated the lag period, although to a smaller extent (Figure 5H). This compound cannot act as an *o*-diphenolic substrate and therefore this effect has to be attributed to

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reduction of dopaquinone to dopa, which can then act as the activator. Lack of such an effect in the case of kaempferol has to be attributed to its strong inhibition of the monophenolase activity. Under conditions applied in our experiments the reaction rates were so small, that the amount of dopa formed during the measurement period was not sufficient to eliminate the lag period, which may be considered as extended indefinitely (to the end of the measurement period).

Oxidation of flavonoids by *o*-quinones also explains the accelerated degradation of quercetin in the presence of catalytic amounts of L-dopa observed previously.[12] Based on the fact that glutathionyl adducts were detected when quercetin was oxidized by peroxidase[40] it was concluded that dopaquinone formed Michael-type adducts with the oxidation products of quercetin.[12] Our results indicate, however, that in the presence of L-dopa quercetin and other flavonoids containing a catechol or conjugated phenolic groups in rings B and C may be oxidized by dopaquinone, just as they are by 4-*tert*-butyl-*o*-quinone and 4-methyl-*o*-quinone. Therefore, in such reactions L-dopa acts as a redox shuttle between the enzyme and the flavonoids in solution (Figure 7). Under such conditions no dopachrome is formed until all the antioxidant flavonoid is exhausted, if the rate of the redox exchange reaction is bigger than the enzymatic oxidation of dopa and the cyclization of dopaquinone (see e.g., Figure 3A and F). We have previously described this phenomenon for a synthetic catechol-containing phosphonic amino acid[41] and such reactions have also been reported by others, e.g., between cyanidin-3-*O*-glucoside and caffeic acid quinone,[42] cyanidin-3-*O*-glucoside and caffeoyltartaric acid quinone,[43] cyanidin-3-*O*-rutinoside and (−)-epicatechin quinone,[44] and catechins and chlorogenic acid quinone.[45] Unfortunately, these reports have gone unnoticed by many researchers testing compounds isolated from natural sources as tyrosinase inhibitors. We hope that the side-reactions described in this paper will be taken into account in interpretation of such results in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01896.

Figures presenting additional experiments and/or data: spectra recorded during oxidation of kaempferol by large amount of tyrosinase (Figure S1), complete sets of spectra recorded during reactions of flavonoids with *o*-quinones (Figure S2), and spectra showing the stability of 4-*tert*-butyl-*o*-benzoquinone and 4-methyl-*o*-benzoquinone (Figure S3) (PDF)

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■ ABBREVIATIONS USED

$DPPH^{\cdot}$,2,2-diphenyl-1-picrylhydrazyl radical; $ABTS^{+\cdot}$,2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical

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