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The influence of Mg(II) and Ca(II) ions on the autoxidation of 4-methylcatechol in weakly alkaline aqueous solutions

Milica G. Nikolić[1] · Nenad S. Krstić[1] · Slavoljub C. Živanović[2] · Goran M. Nikolić[3]

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Abstract

Recent studies indicated that 4-methylcatechol (4-MC) represents an important flavonoid metabolite with various biological activities and potential uses in food industry and that many of those may be connected to its ease of oxidation and/or autoxidation. Ultraviolet–visible (UV–Vis) spectrophotometry and high performance liquid chromatography with diode-array detection (HPLC–DAD) were used to study the influence of Mg(II) and Ca(II) ions on the 4-MC autoxidation in weakly alkaline aqueous solutions. Spectrophotometric data indicated that both Mg(II) and Ca(II) ions greatly enhanced 4-MC autoxidation rate and that autoxidation under conditions applied in this work significantly differs from the enzymatic and periodate oxidation regarding initial products. HPLC–DAD results revealed that, although both investigated ions exert significant catalytic effect on 4-MC autoxidation, Mg(II) ion was about 40% more effective in promoting autoxidation rate due to its larger ionic potential (charge/ionic radius ratio) in comparison to Ca(II) ion. Based on the quantum chemical calculations of the positions of electronic transitions for possible 4-MC autoxidation products and comparison with DAD UV–Vis spectra of chromatographically separated compounds we identified first two autoxidation products to be 2-hydroxy-5-methyl-1,4-benzoquinone and 4-hydroxy-5-methyl-1,2-benzoquinone. Therefore, unlike enzymatic oxidation of 4-MC, where formation of its *o*-quinone is the first step in the reaction, in the systems investigated in this study oxidation to quinones was preceded by the 4-MC hydroxylation. Because the presence of Mg(II) and Ca(II) ions is ubiquitous in all living cells the results of this study may

✉ Milica G. Nikolić
milica.nikolic@pmf.edu.rs

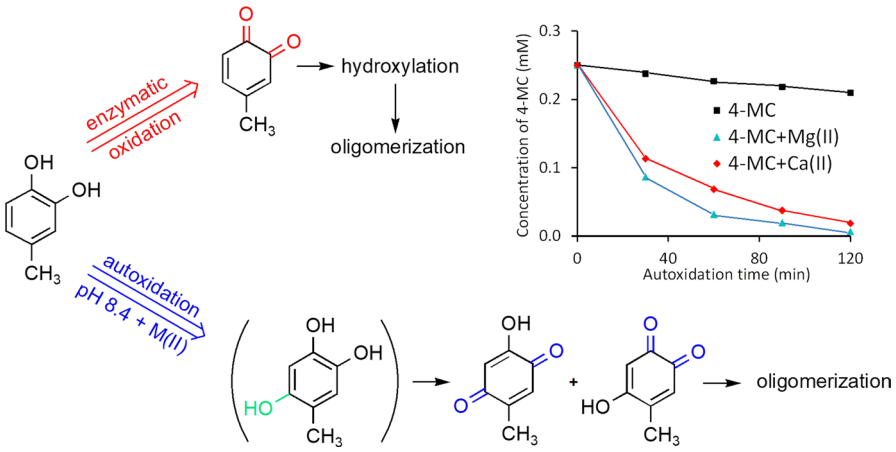
[1] Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia

[2] Research Center for Biomedicine, Faculty of Medicine, University of Niš, Bulevar dr Zorana Đinđića 81, 18000 Niš, Serbia

[3] Department of Chemistry, Faculty of Medicine, University of Niš, Bulevar dr Zorana Đinđića 81, 18000 Niš, Serbia

be important for better understanding of various biological activities and potential applications of 4-MC.

Graphical abstract



Keywords 4-Methylcatechol · Autoxidation · Magnesium · Calcium · Quinones

Introduction

Among the numerous metabolites of flavonoids, 4-methylcatechol (4-MC; also named 3,4-dihydroxytoluene) deserves special attention due to its various biological activities [1, 2]. Early research indicated that 4-MC represents a potent stimulator of nerve growth factor [3] and recent results in rat model showed that 4-MC containing alginate/chitosan hydrogel can be used as a suitable material for peripheral nerve regeneration [4]. Also, 4-MC inhibits inflammatory responses in lipopolysaccharide-activated macrophages [5] and has an inhibitory effect on fatty acid hydroperoxide and hemoglobin dependent lipid peroxidation in Caco-2 cells [6]. It suppresses the progression of nonalcoholic fatty liver disease in mice [7] and UVB-induced wrinkle formation [8], displays potent antiplatelet effects [9] and in a mixture with some other metabolites of quercetin can decrease elevated blood pressure of spontaneously hypertensive rats even in low doses [10]. Besides that, 4-MC can be applied as antioxidant to meat and meat products in order to prevent their oxidation [11, 12]. Many, if not all, of these numerous biological activities and potential applications of 4-MC may be connected to the ease of its oxidation and/or autoxidation [1, 13].

Enzymatic oxidation of 4-MC by tyrosinase [14–16], polyphenol oxidase [17], and laccase [18] was extensively investigated mainly with the goal of enzyme specificity and/or activity determination. Also, enzyme mimicking properties of some metal complexes were studied by using 4-MC as a substrate [19]. Periodate oxidation of 4-MC was investigated either for making a comparison with its enzymatic

oxidation [14, 20] or for studying adducts of its oxidation products with other molecules [21, 22]. On the other hand, the autoxidation of 4-MC was less frequently studied and included ESR analysis of free radicals formed in highly alkaline solutions [15, 23], the study of pH value and Zn(II), Cu(II), and Co(II) ions influence on the 4-MC autoxidation rate [24], and autoxidation in highly alkaline solutions in relation to the crosslinking chemistry with propylamine [21].

Catecholic compounds are generally susceptible to autoxidation in aqueous solutions and their autoxidation rates increase with increasing the pH value [25, 26] and in the presence of metal ions [24, 27, 28]. Although redox active transition metal ions like Mn(II), Fe(II), Cu(II), and Co(II) are more effective in promoting autoxidation of catecholic compounds in comparison to redox inactive metal ions like Ca(II), Mg(II), Al(III), and Zn(II) [24, 27], latter can still enhance the autoxidation at substantial rate [29]. Special attention should be paid to the effect of Mg(II) and Ca(II) ions since they are ubiquitous in the environment, have wide variety of important biological functions, and are far more abundant than transition metal ions in biological systems [30]. Our recent studies clearly demonstrated that Mg(II) and Ca(II) ions greatly affected the autoxidation of natural biologically active catecholic compounds like rutin [31] and caffeic acid [32] in weakly alkaline solutions. Herein we present the results of a similar study on 4-MC since, to the best of authors knowledge, there are no literature data on the Mg(II) and Ca(II) ions influence on the autoxidation of this biologically important compound.

Experimental

Reagents and instruments

4-methylcatechol (≥ 95%) was purchased from Sigma-Aldrich (Germany), trifluoroacetic acid (TFA), $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ (analytical, p.a., grade) were purchased from Merck (Germany), tris(hydroxymethyl)aminomethane (Tris) was purchased from AppliChem (Germany), and hydrochloric acid (HCl) was purchased from Zorka Pharma (Serbia). Deionized water, obtained by TKA Smart2Pure water purification system (Thermo Scientific, Germany), was used for the preparation of all aqueous solutions.

For pH measurements an HI 2214 pH/ORP Meter with a combined glass electrode (Hanna Instruments, USA) was employed.

UV–Vis spectrophotometric data were collected on Evolution 60 scanning spectrophotometer (Thermo Scientific, USA) by recording absorption spectra of solutions placed in 1.0 cm quartz cells. Medium scan rate with the spectral resolution set at 2 nm was applied during the spectra recording.

An Agilent Technologies (USA) 1200 Series system equipped with a vacuum degasser, binary pump, temperature controlled column chamber, autosampler, and a diode-array detector was employed for HPLC analyses. Chromatographic separation was achieved by using 4.6 × 150 mm, 5 µm grain size, Purospher STAR RP-18e column (Merck, Germany) at 30 °C. Gradient elution was employed with 0.1% aqueous solution of TFA (mobile phase A) and acetonitrile (mobile phase B). The gradient of

B was established as follows: 0–12 min from 10 to 40%, 12–15 min from 40 to 80%, 15–20 min from 80 to 90%, 20–23 min from 90 to 10%, 23–25 min holding at 10%. The sample injection volume was 10 µL and the mobile phase flow rate was 0.5 mL/min. The chromatograms were recorded at detection wavelengths of 254, 280, and 330 nm.

Autoxidation procedure

Stock solution of 4-MC (100 mM) was prepared by dissolving exactly weighted mass of 4-MC in methanol. Working solutions of 4-MC (0.5 mM) were prepared just before the mixing with buffer solutions by stock solution dilution with deionized water. Samples for both spectrophotometric and chromatographic measurements were prepared by mixing equal volumes of 4-MC working solution and Tris buffer (100 mM, pH 8.4) without salt addition or with either $MgCl_2$ or $CaCl_2$ addition (200 mM). Reaction mixtures were exposed to air at room temperature (22 ± 1 °C) throughout the experiments. Autoxidation time of 4-MC was measured from the moment of mixing.

Mathematical procedures

Multivariate curve resolution-alternating least squares (MCR-ALS) method [33] was used for the analysis of UV–Vis spectra recorded during the 4-MC autoxidation in order to obtain the number of absorbing species in equilibrium and their spectral and concentration profiles. MATLAB (MathWorks Inc., USA, version R2013a) environment with user-friendly graphical interface [34] was used for all MCR-ALS calculations. Spectral and concentration profiles of absorbing species were calculated under constraints of non-negativity (for concentrations and spectra) and uniformity (for concentrations).

HyperChem software (Hypercube Inc., USA, release 8.0) was used for calculating electron transitions for various compounds which are possible 4-MC autoxidation products. Semi-empirical ZINDO/S quantum chemical calculation method was applied with the default value of σ–σ overlap weighting factor ($OWF_{\sigma-\sigma} = 1.276$) while π–π overlap weighting factor ($OWF_{\pi-\pi}$, default value is 0.585) was adjusted in order to obtain the best match with the positions of absorption maxima in experimental DAD UV–Vis spectra of particular compounds in the manner described in the literature [35].

Results and discussion

UV–Vis spectra recorded immediately after mixing 4-MC and buffer solutions together with the spectrum of 4-MC aqueous solution are shown on the left panel in Fig. 1 and spectra recorded after 60 min of autoxidation are shown on the right panel in Fig. 1.

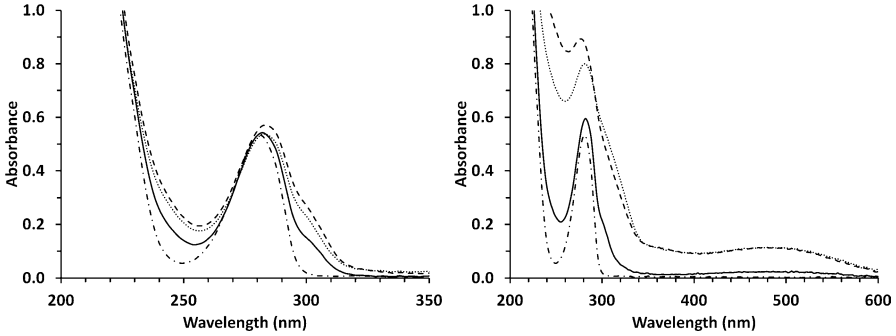


Fig. 1 UV–Vis spectra of 0.25 mM 4-MC aqueous solution (dash-dotted lines) and solutions obtained by mixing 4-MC working solution (0.5 mM) with equal volumes of Tris buffer (pH 8.4) in the absence of metal ions (solid lines) and in the presence of Mg(II) and Ca(II) ions (dashed and dotted lines, respectively). UV–Vis spectra were recorded immediately after mixing (left) and after 60 min of autoxidation (right)

UV–Vis spectra recorded immediately after mixing of 4-MC and buffer solutions displayed only small bathochromic and hyperchromic shifts of the main absorption peak in comparison to 4-MC aqueous solution but also, notable absorption appeared in the area just above 300 nm where no absorption was detected at all in 4-MC aqueous solution. After 60 min of autoxidation in the UV–Vis spectra of all three investigated systems further hyperchromic shift of the main absorption peak and appearance of the new very broad absorption band centered at about 495 nm were observed. These changes were clearly visible in the presence of both Mg(II) and Ca(II) ions while in the system without metal ions they were much less evident. However, these spectral changes were quite different in comparison to the changes observed during the enzymatic [16, 18] and periodate oxidation [20, 22] of 4-MC where appearance of the new absorption band at 400 nm during the initial stages of oxidation indicated that 4-MC *o*-quinone [17, 36] was formed as a first oxidation product. Also, UV–Vis spectral changes observed during the 4-MC autoxidation in more alkaline aqueous solutions (pH 9–12) displayed various patterns [15, 21] but none of them resembled spectral changes detected in this work.

We attempted to extract more information from UV–Vis spectrophotometric data by performing multivariate curve resolution-alternating least squares (MCR-ALS) analysis on the series of spectra recorded during the autoxidation of 4-MC in the presence of Mg(II) and Ca(II) ions at regular time intervals (Fig. S1) in the same manner as we already did for resolving spectral components in the autoxidation of caffeic acid [32] and pyrogallol [37]. However, in the presence of both Mg(II) and Ca(II) ions two spectral components were resolved with the shapes virtually identical to the first and last spectra in series which are shown in Fig. 1 (Fig. S2) and none of them correspond to the UV–Vis spectra attributed to the structures identified as 4-MC oxidation products [17, 36]. Essentially, from the UV–Vis spectrophotometric data we may conclude with absolute certainty that both Mg(II) and Ca(II) ions exert significant catalytic effect on 4-MC autoxidation. It is also clear that the early stages of autoxidation under conditions applied

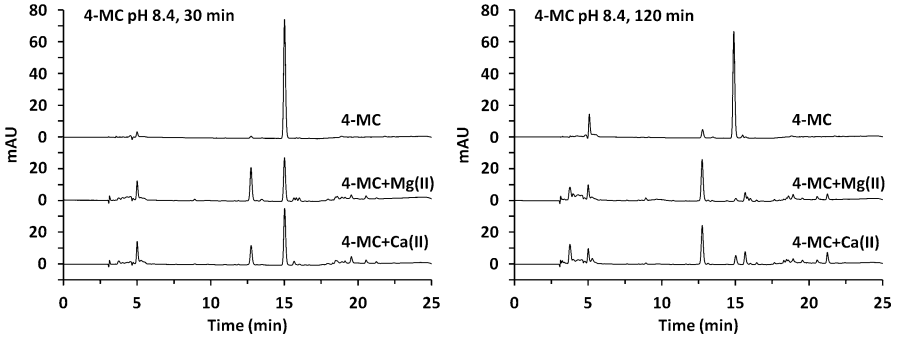


Fig. 2 The chromatograms recorded after 30 and 120 min of 4-MC autoxidation in aqueous solution at pH 8.4 in the absence of metal ions and in the presence of Mg(II) or Ca(II) ions (detection wavelength—280 nm)

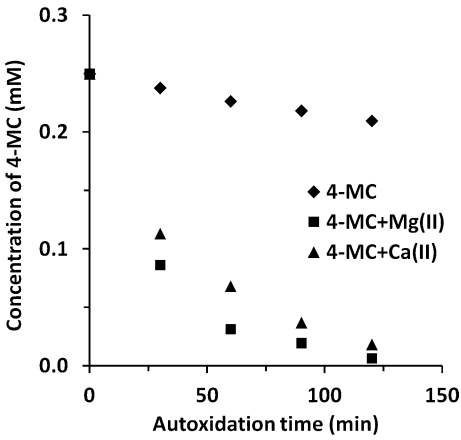


Fig. 3 The changes of 4-MC concentration with time during the autoxidation at pH 8.4 in the absence of metal ions and presence of Mg(II) and Ca(II) ions

in this work significantly differ from both enzymatic and periodate oxidation which is most likely due to the formation of different initial intermediates. For that reason we used HPLC–DAD analysis to get more detailed insight into the mechanism of 4-MC autoxidation in weakly alkaline aqueous solution and the effects of Mg(II) and Ca(II) ions on this process.

The chromatograms recorded after 30 and 120 min of autoxidation for three systems investigated in this study are shown in Fig. 2.

The retention time (RT) of 4-MC is 15.0 min and at first glance it is obvious that its peak intensity decreases much faster in the presence of both investigated ions and this confirms the conclusion drawn from the spectrophotometric data that both Mg(II) or Ca(II) ions, although not redox active, exert significant catalytic effect on 4-MC autoxidation. The changes of 4-MC concentration (measured as the change of its chromatographic peak area) with time during the autoxidation under conditions applied in this work are shown in Fig. 3.

Given that autoxidation was performed under constant O_2 concentration (i.e. reaction mixture was exposed to air during the autoxidation) we analyzed investigated systems as pseudo- first order reactions. The simplest way to handle such data is to use a linearized form of the first order rate law expression:

$$\ln\left(\frac{[4MC]_0}{[4MC]}\right) = k_1' t$$

Here $[4MC]_0$ and [4MC] represent initial and actual concentrations of 4-MC, k_1' represents the pseudo-first order rate constant, and t is autoxidation time. Kinetic parameters obtained by the linear least squares fitting (Fig. S3) are given in Table 1.

Data shown in Table 1 reveal that both investigated ions greatly increased 4-MC transformation rate which is the consequence of additional deprotonation of hydroxyl groups in catecholic substances in the presence of alkaline-earth ions [29]. In the presence of Mg(II) ions this process proceeds at about 40% faster rate than in the presence of Ca(II) ions and this may be explained by the larger ionic potential (charge/ionic radius ratio) of Mg(II) ion in comparison to Ca(II) ion which causes stronger Mg(II) ion-ligand interactions [38].

Another, more accurate, way for obtaining kinetic parameters in such cases is the use of the non-linear least squares fitting of untransformed data [39]. Application of non-linear least squares fitting to our experimental data was performed by using Excel file kindly provided by Prof. Gábor Lente at http://lenteg.ttk.pte.hu/KinetFit.html. Obtained values of pseudo-first order rate constants for the 4-MC autoxidation in the presence of Mg(II) and Ca(II) ions (0.0371 ± 0.00164 and 0.0266 ± 0.00281, respectively) are higher than values shown in Table 1 (0.0303 ± 0.00104 and 0.0217 ± 0.00046, respectively) and recalculated values also show that 4-MC autoxidation in the presence of Mg(II) ions proceeds at about 40% faster rate than in the presence of Ca(II) ions. However, graphical comparison of linear and non-linear least squares fitting of our experimental data (Fig. S4) as well as slightly better values for correlation coefficients (0.9945 vs. 0.9937 in the case of 4-MC autoxidation in the absence of metal ions, 0.9993 vs. 0.9848 in the presence of Mg(II) ion, and 0.9966 vs. 0.9944 in the presence of Ca(II) ion) indicate that non-liner least squares fitting should be used [40], especially in the similar cases where no great differences are expected between the systems under study [26].

From the chromatograms shown in Fig. 2 we may assume that first autoxidation products in the systems investigated in this study are two compounds with retention

Table 1 Kinetic parameters for the autoxidation of 4-MC at pH 8.4 obtained by the linear least squares fitting

System	Pseudo-first order rate constant, k_1' (min^{-1})	Half-time of the reaction, $t_{1/2}$ (min)	Correlation coefficient, r^2
4-MC	0.0015 ± 0.00003	462.1	0.9937
4-MC + Mg(II)	0.0303 ± 0.00104	22.9	0.9848
4-MC + Ca(II)	0.0217 ± 0.00046	31.9	0.9944

time values of 5.0 and 12.7 min. This assumption is justified by their concentration changes with time which are shown in Fig. 4. Also, as in the case of catechol [41], both compounds which are supposed to be the first 4-MC autoxidation products have retention time values lower than the retention time value of 4-MC which means that they are more polar than 4-MC since reversed phase system was used for chromatographic separation.

Somewhat similar patterns of concentration change with time for these first two products of 4-MC autoxidation in the presence of Mg(II) and Ca(II) ions, i.e., rapid concentration increase during the first 30 min of autoxidation with smaller subsequent changes, may also explain why MCR-ALS analysis of spectrophotometric data gave very similar results for the influence of Mg(II) and Ca(II) ions on this process (Fig. S2).

DAD UV–Vis spectra of compounds with retention time values of 5.0 and 12.7 min, together with the DAD UV–Vis spectrum of 4-MC, are shown in Fig. 5.

Unlike 4-MC, its first autoxidation products have absorption maxima far above 300 nm which may be characteristic for quinoid compounds [42], but none of their DAD UV–Vis spectra matches the UV–Vis spectrum of 4-MC *o*-quinone [17, 36]

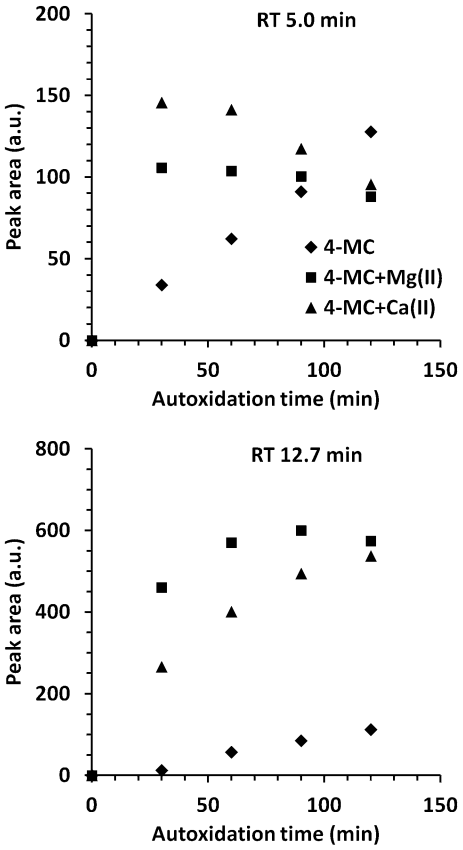


Fig. 4 Concentration changes with time for the first two products formed during the 4-MC autoxidation at pH 8.4 in the absence of metal ions and in the presence of Mg(II) or Ca(II) ions

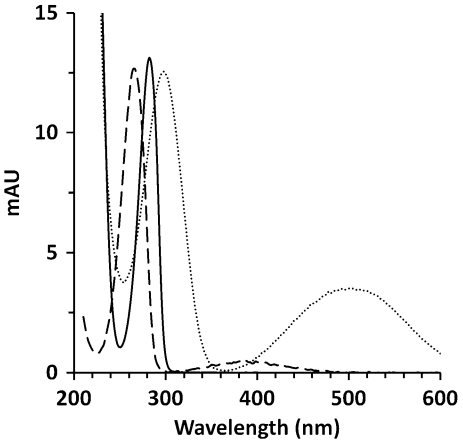


Fig. 5 DAD UV–Vis spectra of 4-MC (solid line) and compounds formed during the 4-MC autoxidation at pH 8.4 and chromatographically detected at retention time values of 5.0 (dotted line) and 12.7 min (dashed line)

which was found to be the first oxidation product of 4-MC during its enzymatic oxidation [16–18]. On the other hand, the DAD UV–Vis spectrum of compound with retention time value of 12.7 min almost perfectly matches UV–Vis spectrum of the compound obtained during the 4-MC oxidation by polyphenol oxidase and identified as hydroxylated *p*-quinone [17] which is one of the five possible quinone structures derived from hydroxylated 4-MCs (Fig. S5). Also, positions of electronic transitions obtained by quantum chemical calculations for this hydroxylated *p*-quinone structure are in very good agreement with the positions of absorption maxima in the experimental DAD UV–Vis spectrum of compound with the retention time value of 12.7 min (Fig. S6).

We further performed quantum chemical calculations of electronic transitions for remaining four possible quinone structures derived from hydroxylated 4-MCs and reasonable agreement of electronic transitions positions with the positions of absorption maxima in the experimental DAD UV–Vis spectrum of compound with the retention time value of 5.0 min was obtained only for 4-hydroxy-5-methyl-1,2-benzoquinone (Fig. S7). Although in this case calculated wavelength values for electronic transitions are about 20 nm lower than the positions of absorption maxima in the experimental DAD UV–Vis spectrum, such an extent of disagreement is not uncommon for *o*-quinone structures, even when far more elaborate and time consuming calculation methods like DFT are used [43].

An additional argument in favor of our assignment of specific structures to the first autoxidation products of 4-MC is that order of calculated logP values (from two different sources) for 4-MC and two proposed structures matches the order of their retention time values (Table S1). In a reversed phase chromatographic system used in this study lower retention time values correspond to the lower logP values (more polar compounds) [44] but because gradient elution was applied only qualitative correlation is possible.

Based on the previous discussion we concluded that, unlike enzymatic and periodate oxidations of 4-MC which start with *o*-quinone formation, the 4-MC autoxidation under conditions applied in this work starts with the hydroxylation of 4-MC and

very fast subsequent quinone(s) formation. We ruled out the possibility that 4-MC *o*-quinone formation with the very fast subsequent transformation to hydroxylated products preceded direct 4-MC hydroxylation because our spectrophotometric data (Fig. S1) did not show any presence of the well resolved peak at ~ 400 nm characteristic for 4-MC *o*-quinone [36] easily observable during the 4-MC oxidation by various oxidants [16, 18–20, 22]. Also, while chromatographic peaks with DAD UV–Vis spectrum virtually identical to the UV–Vis spectrum of 4-MC *o*-quinone were detected during the 4-MC oxidation with apple polyphenol oxidase [17], no such a chromatographic peak was found in the systems investigated in this work,

Hydroxylation of 4-MC was much easier in the presence of Mg(II) and Ca(II) ions due to the metastable complex formation between alkaline-earth ions and *ortho* semiquinone anion radicals of catecholic substances which is usually referred to as ″spin stabilization″ [29]. Mechanism of the first relatively stable intermediates formation during the autoxidation of 4-methylcatechol in the presence of Mg(II) and Ca(II) ions is shown in the scheme given on Fig. 6.

As shown in Fig. 4, maximum concentrations of the first 4-MC autoxidation products were reached after some autoxidation time and their concentration decrease indicated the formation of other autoxidation products as evidenced by the appearance and intensity increase with time of chromatographic peaks with retention time values greater than 15.0 min. According to the LC–MS data found in the literature for the reversed phase chromatographic separation in 4-MC oxidation model, retention time values greater than retention time of 4-MC are characteristic for the products formed by its dimerization and oligomerization [45]. Evidence for the formation of dimeric and oligomeric 4-MC products with greater retention time values was also given in the literature for its oxidation by polyphenol oxidase [17]. Indeed, polymers produced by peroxidase-catalyzed oxidation of simple phenols, including 4-MC, were studied with the aim of designing new bioinspired antioxidants for application in food, biomedicine and material sciences [46]. Comparative display of reaction pathways for enzymatic oxidation and autoxidation of 4-MC in the presence of Mg(II) and Ca(II) ions is shown in Fig. 7.

Results obtained in this work suggest that studies of various 4-MC biological activities should consider its possible interactions with Mg(II) and Ca(II) ions, especially because there already exists some evidence that 4-MC may interfere with calcium intracellular signaling [9]. Also, further investigations of 4-MC potential uses in food industry should take into account its possible interactions with Mg(II) and Ca(II) ions in addition to the effects of light and oxygen [13]. Another important

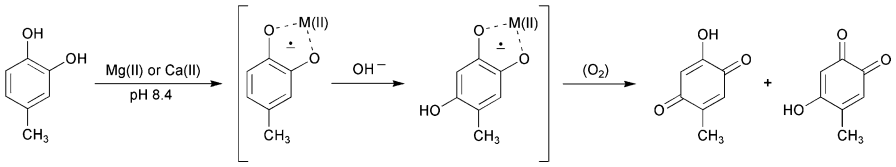


Fig. 6 Schematic representation of the first relatively stable intermediates formation during the autoxidation of 4-methylcatechol in the presence of Mg(II) and Ca(II) ions at pH 8.4

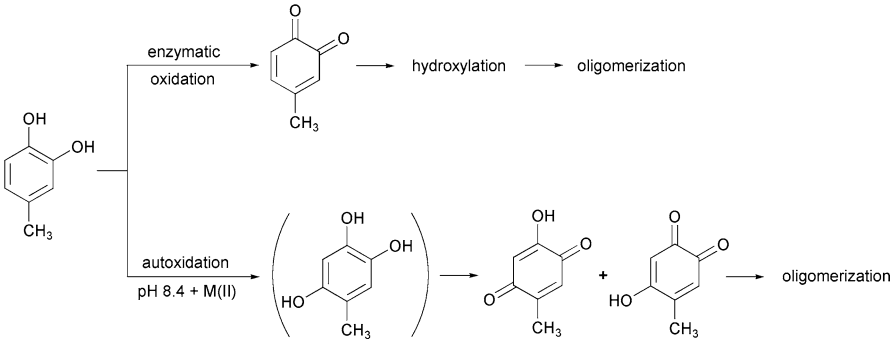


Fig. 7 Comparative display of reaction pathways for enzymatic oxidation and autoxidation of 4-MC at pH 8.4 in the presence of Mg(II) and Ca(II) ions

point to note is that two quinones were identified as first relatively stable intermediates during the 4-MC autoxidation. In the first place, this may be significant because quinones are known to be reactive compounds with numerous biological effects which could be toxic but also sometimes equivocal or even beneficial [47]. In addition, due to their ability to react with nucleophiles, quinones may form covalent adducts with amino acids, peptides, and proteins in food and thus alter their physicochemical properties and nutritive values [48].

Conclusions

UV–Vis spectrophotometric and HPLC–DAD analysis of the 4-MC autoxidation in weakly alkaline aqueous solutions showed that this process proceeds at very slow rate in the absence of metal ions. The presence of Mg(II) and Ca(II) ions greatly increased the 4-MC autoxidation rate with Mg(II) ion being about 40% more effective due to its larger ionic potential (charge/ionic radius ratio) in comparison to Ca(II) ion. Both spectrophotometric and chromatographic data indicated that the mechanism of 4-MC autoxidation in weakly alkaline aqueous solutions differs from the mechanism of its enzymatic or periodate oxidation. Analysis of DAD UV–Vis spectra for individual chromatographic peaks in combination with quantum chemical calculations allowed us to identify two initial products of 4-MC autoxidation. Identified structures indicated that initial steps in the 4-MC autoxidation under conditions applied in this work are its hydroxylation and very fast subsequent oxidation of hydroxylated 4-MC to 2-hydroxy-5-methyl-1,4-benzoquinone and 4-hydroxy-5-methyl-1,2-benzoquinone while 4-MC *o*-quinone, which is the first oxidation product in enzymatic and periodate oxidation of 4-MC, was not detected at all. Chromatographic data also indicated formation of dimeric and oligomeric products of 4-MC transformation at longer autoxidation times. The results of this study may contribute to the better understanding of various biological activities and potential uses of 4-MC because the presence of Mg(II) and Ca(II) ions is ubiquitous in all living cells and environment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11144-022-02180-3.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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