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Original article

The Influence of Mg(II) and Ca(II) Ions on Rutin Autoxidation in Weakly Alkaline Aqueous Solutions

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SUMMARY

Rutin (quercetin-3-O-rutinoside) is one of the most abundant bioflavonoids with various biological and pharmacological activities. Considering the ubiquitous presence of Mg(II) and Ca(II) ions in biological systems we decided to investigate their influence on the autoxidation of rutin in weakly alkaline aqueous solutions. Changes in UV-Vis spectra recorded during the rutin autoxidation in aqueous solution at pH 8.4 revealed that this process was very slow in the absence of metal ions. The presence of Mg(II) and, especially Ca(II) ion, increased the transformation rate of rutin. UV-Vis spectra recorded after prolonged autoxidation indicated the formation of humic acid-like products in the presence of Mg(II) and Ca(II) ions. Four new compounds formed during the initial stage of rutin autoxidation in the presence of Mg(II) and Ca(II) ions were detected by HPLC-DAD. Based on the analysis of their DAD UV-Vis spectra and comparison of their retention times with the retention time value for rutin, we concluded that the initial rutin transformation products were formed by the water addition on double bond in ring C and hydroxylation of ring B. A very small decrease of the initial rutin concentration (4%) was observed by HPLC-DAD in the absence of metal ions for the period of 90 minutes. However, rutin concentration decrease was much larger in the presence of Mg(II) and Ca(II) ions (14% and 24%, respectively). The more pronounced effect of Ca(II) ion on the rutin autoxidation may be explained by the stronger binding of Mg(II) ion to rutin and thus greater stabilizing effect on reaction intermediates caused by its higher ionic potential (charge/ionic radius ratio) in comparison to Ca(II) ion. The results of this study may contribute to the better understanding of interactions of Mg(II) and Ca(II) ions with natural phenolic antioxidants which are important for their various biological activities.

Key words: **rutin, autoxidation, magnesium, calcium**

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INTRODUCTION

Phenolic compounds are ubiquitous in plants and their dietary intake has many beneficial effects on human health (1, 2). Rutin (quercetin-3-O-rutinoside), the glycoside between the flavonol quercetin and disaccharide rutinose (α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose) (Figure 1) is one of the most abundant bioflavonoids with various biological and pharmacological activities (2, 3).

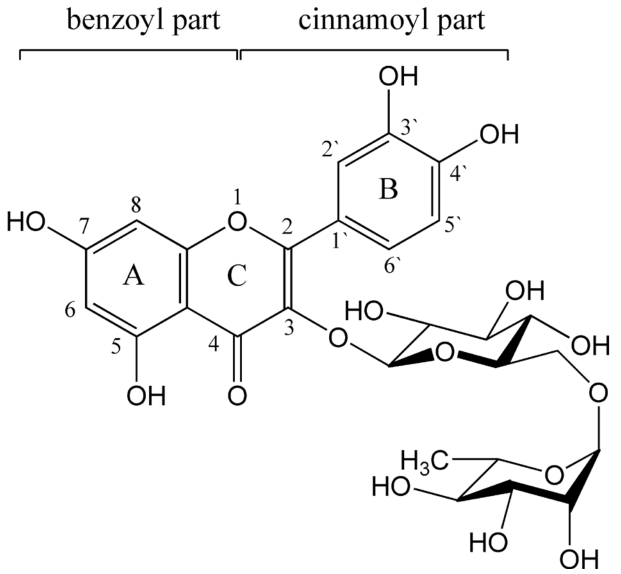

Figure 1: **Structural formula of rutin with atom numbering and labeling of structural units responsible for the main absorption bands in UV-Vis spectra**

Oxidation or/and autoxidation of natural phenolic compounds may have profound influence on their biological activity and stability of certain types of foods (4). Compounds having two or three vicinal hydroxyl groups on the same benzene ring are especially susceptible to autoxidation, and it was found that this process is mainly influenced by the pH value (4) and the presence of transition metal ions like Mn(II), Fe(II), and Cu(II) (5, 6) which are redox active and strongly enhance the autoxidation process. However, the influence of Mg(II) and Ca(II) ions on the autoxidation of natural phenolic compounds was studied less frequently (5, 7), although these two ions are far more abundant in living organisms than transition metal ions and have a wide variety of important biological functions (8).

There are literature data on the enzymatic (9-12), chemical (11, 13-15), and electrochemical (16, 17) oxidation of rutin, but its autoxidation was not studied in detail except in the study of the effect of thermal processing on rutin and quercetin in aqueous solutions (18). Considering the biological importance of both rutin and Mg(II) and Ca(II) ions we found it important to study the influence of Mg(II) and Ca(II) ions on the rutin autoxidation in a weakly alkaline aqueous solution as a model system.

MATERIAL AND METHODS

All the chemicals used in this study were of analytical (p.a.) grade, except acetonitrile (ACN) which was of HPLC grade quality. Rutin was purchased from Sigma Aldrich (Germany) and trifluoroacetic acid (TFA) was purchased from Merck (Germany). Deionized water was obtained by using TKA Smart2-Pure water purification system (Thermo Scientific, Germany).

Working solutions of rutin (0.5 mmol dm^{-3}) were prepared just before the use by dissolving exactly weighted amount of rutin in deionized water. Autoxidation of rutin was initiated by mixing equal volumes of rutin solution and Tris buffer (100 mmol dm^{-3}, pH 8.4) without salt addition or with either $MgCl_2$ of $CaCl_2$ addition (0.2 mol dm^{-3}). Autoxidizing solutions were kept at room temperature (22 ± 1°C) exposed to air during experiments.

An Evolution 60 UV-Vis scanning spectrophotometer (Thermo Scientific, USA) with 1.0 cm quartz cells was employed for spectrophotometric measurements. UV-Vis spectra were recorded immediately after mixing rutin and Tris buffer solutions and at regular time intervals of 15 minutes for the next 120 minutes. After that, prolonged autoxidation of rutin was followed by recording UV-Vis spectra at various time intervals up to 24 hours.

An Agilent Technologies 1200 Series system equipped with a vacuum degasser, binary pump, temperature controlled column chamber, autosampler, and a diode-array detector (DAD) was employed for HPLC analyses. Separation was achieved by using Purospher STAR RP-18e column (150×4.6 mm, 5 μm, Merck, Germany) at 30°C. Gradient elution was employed with 0.1% aqueous trifluoroacetic acid (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%; 15-16 min holding 80%, and 16-20 min from 80% to 10%. The sample injection volume was 10 μL and the mobile phase flow rate was 0.5 mL/min. The chromatograms were recorded at a detection wavelength of 254 nm (typical for benzoic acid and related compounds).

RESULTS AND DISCUSSION

UV-Vis spectra recorded immediately after mixing rutin solution with Tris buffer in the absence of metal ions and in the presence of Mg(II) and Ca(II) ions are shown in Figure 2.

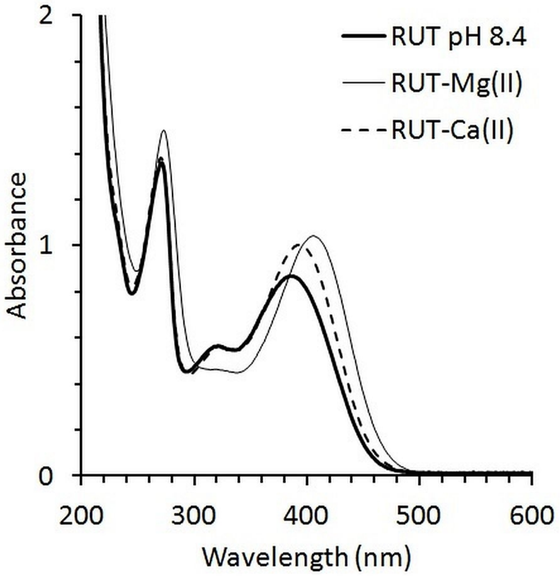

Figure 2: **UV-Vis spectra of rutin recorded immediately after mixing of rutin solution with Tris buffer pH 8.4 in the absence of metal ions and in the presence of Mg(II) and Ca(II) ions**

Two absorption bands characteristic for flavonoids, the benzoyl band in the 240-280 nm range and the cinnamoyl band in the 320-385 nm range (19) , appear in the spectra shown in Figure 2. However, at pH 8.4 in the absence of metal ions absorption maxima of these bands shifted to higher wavelengths (271 and 385 nm) in comparison to rutin in acidic solutions (256 and 356 nm at pH 3). This bathochromic shift of absorption bands was caused by the partial deprotonation of rutin molecule with increasing pH value of solution as in the case of similar flavonoid molecules (20). Complexation of flavonoids with metal ion causes further bathochromic shift of characteristic absorption bands (19), and in the presence of Mg(II) and Ca(II) ions absorption maximum of the cinnamoyl band shifted to 406 nm and 395 nm, respectively, while there was almost no shift of the benzoyl band. As in the case of quercetin complexation with Mg(II) and Ca(II) ions (21), larger bathochromic shift was caused by the presence of Mg(II) ion which may be explained by its higher ionic potential (charge/ionic radius ratio) in comparison to Ca(II) ion.

UV-Vis spectra recorded at regular 15 minutes time intervals for the period of 120 minutes for mixtures of rutin solution and Tris buffer in the absence of metal ions and in the presence of Mg(II) and Ca(II) ions are shown in Figure 3.

Very small, practically negligible changes occurred in the UV-Vis spectra during the autoxidation of rutin in aqueous solution at pH 8.4 in the absence of metal ions for the period of 120 minutes. On the other side, in the presence of Mg(II), and especially Ca(II) ion, notable changes occurred in the UV-Vis spectra. There was absorbance decrease for the two main absorption bands but also small absorbance increase at some wavelengths (indicated by the arrows in Figure 3). In the rutin-Ca(II) system, three well defined isobestic points appeared at 262, 278, and 306 nm, thus indicating the existence of two absorbing species in equilibrium under these conditions. Changes in UV-Vis spectra observed during the autoxidation of rutin in the presence of Mg(II) and Ca(II) ions were quite different in comparison to the changes occurring during the irradiation and thermolysis of rutin where the overall decrease of absorption at all wavelengths indicated the formation of degradation products with no chromophore structures (22).

UV-Vis spectra recorded after 20 hours of rutin autoxidation in the absence and presence of metal ions are shown in Figure 4.

UV-Vis spectrum recorded after 20 hours of rutin autoxidation in the absence of metal ions at pH 8.4 was almost identical to its initial UV-Vis spectrum with only a small decrease of absorbance for the two main absorption bands. In the case of rutin autoxidation in the presence of Mg(II) and Ca(II) ions, UV-Vis spectra recorded after 20 hours of autoxidation have much different appearance in comparison to the initial spectra and resemble the UV-Vis spectra of products obtained during the oxidation of rutin by horseradish peroxidase (10) and spectra of humic acids (natural compounds formed by the polymerization of simple phenolic compounds) (23).

Further investigation of the rutin autoxidation process was performed by HPLC-DAD. Chromatograms were recorded at regular 30-minute intervals from the start of autoxidation, and beside the decrease of rutin chromatographic peak intensity no significant new compounds were observed after 60 minutes of

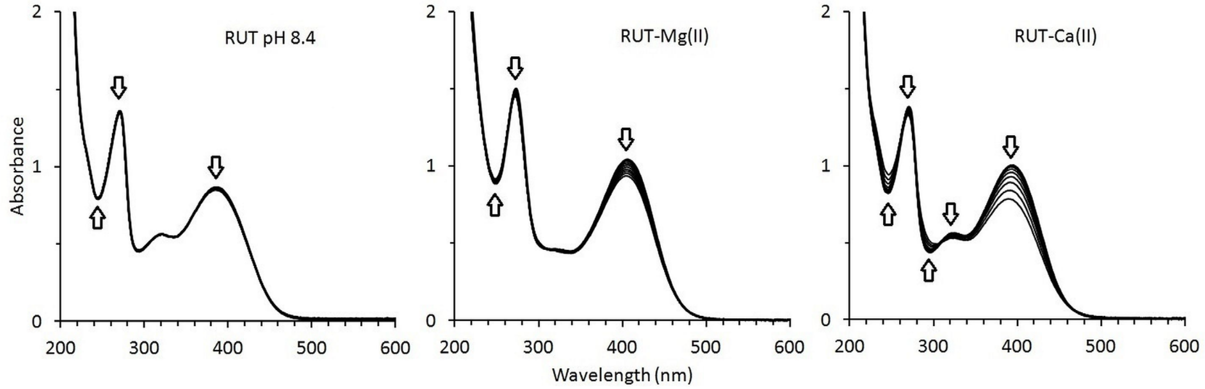

Figure 3: **UV-Vis spectra recorded during the rutin autoxidation at regular 15 minute intervals for the period of 120 minutes. Arrows indicate changes of absorbance at specific wavelengths**

autoxidation. Chromatograms recorded after 90 minutes of rutin autoxidation in the absence and presence of metal ions are shown in Figure 5.

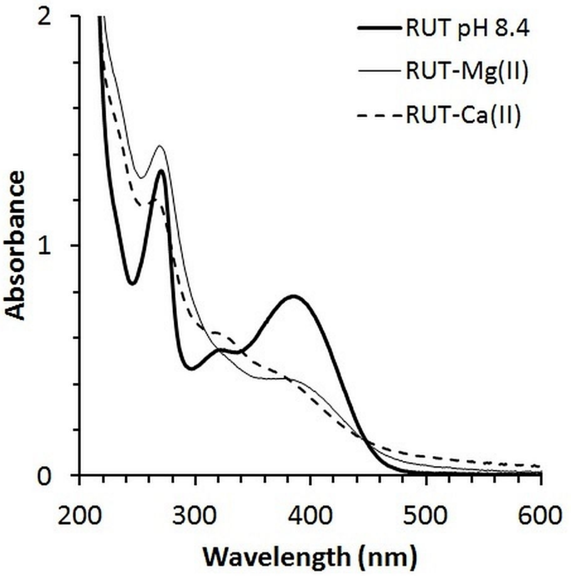

Figure 4 **:UV-Vis spectra recorded after 20 hours of rutin autoxidation at pH 8.4 in the absence of metal ions and in the presence of Mg(II) and Ca(II) ions**

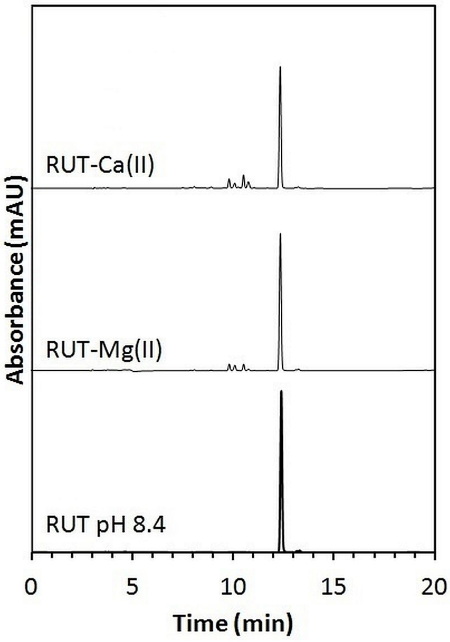

Figure 5: **Chromatograms recorded after 90 minutes of rutin autoxidation at pH 8.4 in the absence of metal ions and in the presence of Mg(II) and Ca(II) ions. Detection wavelength was set at 254 nm**

In the chromatogram recorded after 90 minutes of rutin autoxidation in aqueous solution at pH 8.4 in the absence of metal ions, only the peak originating from rutin at retention time (RT) of 12.3 minutes can be observed. The peak originating from rutin is still prevailing in chromatograms recorded after 90 minutes of rutin autoxidation in the presence of Mg(II) and Ca(II) ions, but four minor peaks at RT 9.8, 10.1, 10.5, and 10.8 minutes originating from the products of rutin transformations in these systems appeared. Smaller RT values in comparison to rutin indicate that these new compounds are more polar because nonpolar C18 column was used for chromatographic separation. DAD UV-Vis spectra extracted from the chroma-tographic peaks of these new compounds are identical for both RUT-Mg(II) and RUT-Ca(II) systems and very similar to the DAD UV-Vis spectrum of rutin. Beside this, DAD UV-Vis spectra of compounds with RT values of 9.8 and 10.5 minutes are practically identical (absorbance ratios of their spectra are nearly constant in the wavelength range from 210 to 400 nm). Plausible explanation for the identical UV-Vis spectra of two compounds with different RT values may be that they are diastereoisomers formed by the addition of water to the double bond between C2 and C3 in the ring C of rutin (Figure 1). The possibility of solvent addition on this double bond during the oxidation by DPPH or autoxidation of quercetin and its derivatives (including rutin) in protic solvents has been already reported in the literature (24). For the other two minor compounds obtained during the autoxidation of rutin in the presence of Mg(II) and Ca(II) ions appearing in chromatograms at RT 10.1 and 10.8 minutes, we tentatively propose the structures obtained by the hydroxylation of ring B of rutin. This assumption is based on the fact that the possibility of B ring hydroxylation in alkaline solutions is well documented for some flavonols with two *ortho¬* hydroxyl groups on this ring (25). Also, as already stated, smaller RT values for these compounds in comparison to rutin (RT 12.3 minutes) indicate that they are more polar. However, RT value differences are not so large as to indicate some big structural changes like ring cleavage with very polar carboxylic group formation.

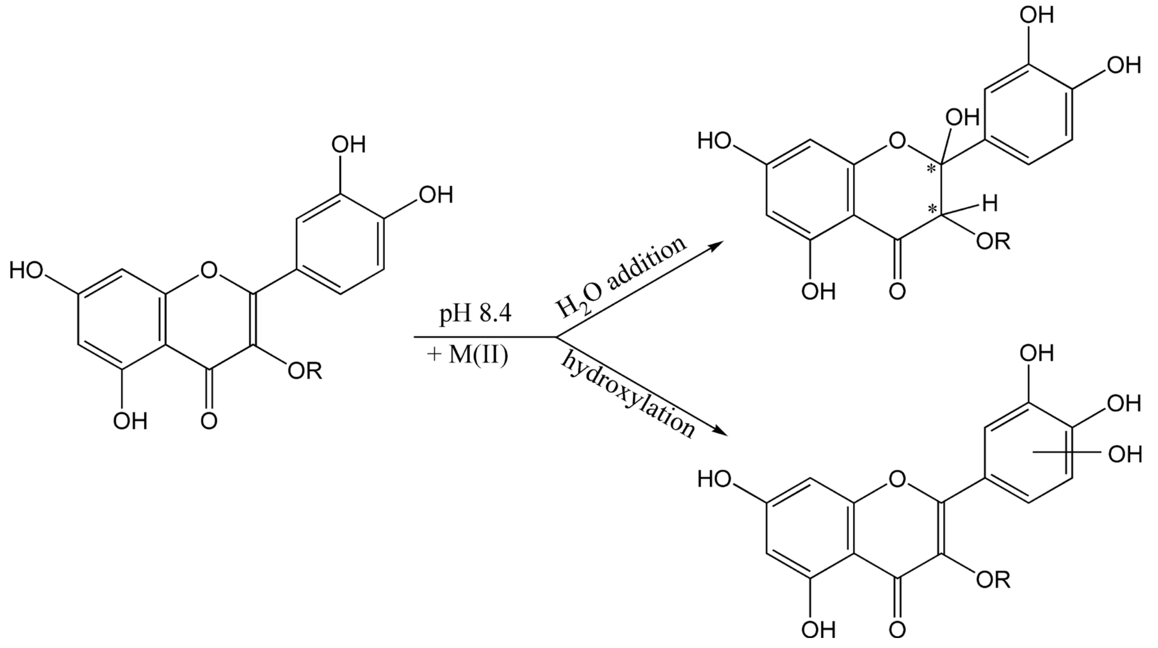

***Figure 6*: Schematic presentation of initial rutin transformations during its autoxidation at pH 8.4 in the presence of Mg(II) and Ca(II) ions (R = rutinoside).**

Possible transformations of rutin during the initial phase of autoxidation in weakly alkaline aqueous solution in the presence of Mg(II) and Ca(II) ions are schematically presented in Figure 6.

Measurements of rutin HPLC peak area at various time intervals allowed the estimation of rutin concentration change during autoxidation in systems studied in this work, and these results are shown in Figure 7.

Rutin concentration decreased only about 4% for 90 minutes during the autoxidation at pH 8.4 in the absence of metal ions, but the presence of Mg(II), and especially Ca(II) ion, significantly increased its consumption rate. About 14% and 24% decrease of initial rutin concentration was observed during its autoxidation in the presence of Mg(II) and Ca(II) ions, respectively, for the period of 90 minutes. The increased transformation

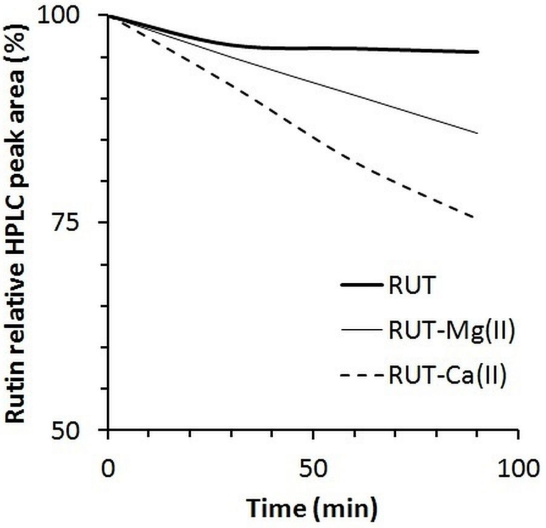

***Figure 7*: Relative change of HPLC peak area of rutin with time during its autoxidation at pH 8.4 in the absence of metal ions and in the presence of Mg(II) and Ca(II) ions.**

rate of rutin in the presence of Mg(II) and Ca(II) ions may be explained by the increased dissociation constants of hydroxyl groups of phenolic compounds in the presence of divalent diamagnetic metal ions (26), which promotes further transformations. However, although both metal ions increased the rutin transformation rate, the effect of Ca(II) ion was more pronounced probably because of tighter binding of Mg(II) ion to rutin and greater stabilizing effect on reaction intermediates.

CONCLUSION

UV-Vis spectrophotometry and HPLC-DAD have been applied for studying the influence of Mg(II) and Ca(II) ions on the autoxidation of rutin in weakly alkaline aqueous solutions. Changes in UV-Vis spectra recorded during the rutin autoxidation in aqueous solution at pH 8.4 revealed that this process was very slow in the absence of metal ions. The presence of Mg(II) and especially Ca(II) ion, increased the transformation rate of rutin and this may be explained by the increased dissociation of phenolic hydroxyl groups which promotes further transformations. UV-Vis spectra recorded after prolonged autoxidation (20 hours) indicated the formation of humic acid-like products in the presence of Mg(II) and Ca(II) ions. HPLC-DAD enabled the detection of four new compounds formed during the rutin autoxidation in the presence of Mg(II) and Ca(II) ions. Based on the analysis of DAD UV-Vis spectra of these newly formed compounds and comparison of their retention times with the retention time value for rutin, we concluded that the initial rutin transformation products were obtained by the water addition on double bond in ring C and hydroxylation of ring B. HPLC-DAD also enabled the quantitative estimation of rutin consumption during autoxidation in the absence of metal ions and in the presence of Mg(II) and Ca(II) ions. Very small decrease in the initial rutin concentration (4%) was detected in the absence of metal ions for the period of 90 minutes; however, rutin concentration decrease was much larger in the presence of Mg(II) and Ca(II) ions (14% and 24%, respectively). The more pronounced effect of Ca(II) ion on the rutin autoxidation may be explained by the stronger binding of Mg(II) ion to rutin and thus greater stabilizing effect on reaction intermediates caused by its higher ionic potential (charge/ionic radius ratio) in comparison to Ca(II) ion.

The results of this study may contribute to the better understanding of interactions of Mg(II) and Ca(II) ions with natural phenolic antioxidants which are important for their various biological activities, and also for the optimization of conditions for processing and storage of certain types of foods.

Acknowledgment

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Uticaj Mg(II) i Ca(II) jona na autooksidaciju rutina slabo baznim vodenim rastvorima

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SAŽETAK

Rutin (kvercetin-3-O-rutinozid) je jedan od najzastupljenijih bioflavonoida sa različitim biološkim i farmakološkim aktivnostima. Imajući u vidu univerzalno prisustvo Mg(II) i Ca(II) jona u biološkim sistemima, odlučili smo da ispitamo njihov uticaj na autooksidaciju rutina u slabo baznim vodenim rastvorima.

Praćenjem promena u UV-Vis spektrima koji su snimani u toku autooksidacije rutina u vodenom rastvoru na pH 8,4 utvrđeno je da je ovaj proces veoma spor u odsustvu metalnih jona. Prisustvo Mg(II) i naročito Ca(II) jona povećelo je brzinu transformacije rutina. UV-Vis spektri, snimljeni nakon produžene autooksidacije rutina, ukazali su na formiranje proizvoda sličnih huminskim kiselinama u prisustvu Mg(II) i Ca(II) jona. Četiri nova jedinjenja nastala u početnoj fazi autooksidacije rutina u prisustvu Mg(II) i Ca(II) jona detektovana su pomoću HPLC-DAD. Na osnovu analize njihovih DAD UV-Vis spektara i poređenja njihovih retencionih vremena sa retencionim vremenom za rutin zaključeno je da su početni proizvodi transformacije rutina nastali adicijom vode na dvostruku vezu u prstenu C i hidroksilacijom prstena B. Veoma malo smanjenje početne koncentracije rutina (4%) je uočeno pomoću HPLC-DAD u odsustvu metalnih jona u periodu od 90 minuta, ali je smanjenje koncentracije rutina bilo znatno veće u prisustvu Mg(II) i Ca(II) jona (14%, odnosno 24%). Izraženiji uticaj Ca(II) jona na autooksidaciju rutina može se objasniti jačim vezivanjem Mg(II) jona za rutin, što doprinosi stabilizaciji reakcionih intermedijera, a uzrokovano je većim jonskim potencijalom (odnos naelektrisanje/jonski radijus) u odnosu na Ca(II) jon.

Rezultati ovog istraživanja mogu da doprinesu boljem razumevanju interakcija Mg(II) i Ca(II) jona sa prirodnim fenolnim antioksidantima koje su značajne za njihove različite biološke aktivnosti.

Ključne reči: **rutin, autooksidacija, magnezijum, kalcijum**