Comparative spectrophotometric determination of 3-hydroxyflavone based on zinc and aluminium complexes and their antioxidative profiles

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Abstract

Flavonoids, as plant-derived compounds, were essential active components in traditional medicine for centuries. Their potential or confirmed effects include antiviral, antimutagenic, anti-inflammatory, antibacterial, vasodilatory, and anticancer properties. The promotion of a plant-based diet, along with the benefits of consuming flavonoids, has recently become increasingly attractive. 3-Hydroxyflavone (3HF) is the structural spine of flavonols, an important subgroup of flavonoids. Although 3HF itself does not exist in plants per se, it exerts many of its effects because of its characteristics that allow it to prevent free radical generation. This work is focused on the characterization of 3HF complexes with zinc(II) and aluminium(III) ions (Zn-3HF and Al-3HF, respectively). Besides this, a simple, fast, and low-priced spectrophotometric method for 3HF determination, with very low LOD and LOQ, based on Zn-3HF and Al-3HF formation, was established. A slight advantage is given to the modification with Al³⁺ ion on pH 4.91, due to very low LOD values of $1.83 \times 10^{-7}$ molL⁻¹, and $5.50 \times 10^{-7}$ molL⁻¹, respectively, and a high correlation coefficient, $R = 0.99986$. Furthermore, the antioxidant ability of Zn-3HF, Al-3HF, and parent 3HF was examined by the ABTS and DPPH tests. They brought the Zn-3HF complex to the fore as a potential antioxidative agent.

Key words: spectrophotometry, 3-hydroxyflavone, zinc complex, aluminium complex, antioxidative capacity

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Introduction

1. Flavonoids

Herbal drugs are the oldest form of medicines and are used to treat many diseases. This kind of application is possible because of their active ingredients. For some herbal drugs, the chemical nature of the active ingredients is unknown; however, for a large number of compounds isolated from plants, the structure has been defined, and their action has been confirmed. Pharmacologically active ingredients of plants are classified as secondary plant metabolites. One of those ingredients are flavonoids (1).

Flavonoids are extensively spread in plants. So far, more than 8,000 flavonoids have been isolated from plants, and this number is still growing. As the structure of flavonoids became more complex and diverse with the evolution of plants, their role in plants became increasingly important (2). They represent the most important plant pigments; if they are not colored by themselves, they appear as co-pigments. Thus, colorless flavones and flavonols complement the color of anthocyanins. Due to the characteristic absorption spectrum, they help plants attract insects. As integral parts of plant enzyme systems, they are responsible for developing metabolic processes. In addition, they prevent infections of plant tissue by bacteria, yeasts, or viruses (phytoalexin function), and protect plants from excessive UV radiation (1).

Besides great importance for plants, flavonoids are significant for animals and humans. The effects they exhibit are primarily based on their antioxidant action, and it has been unequivocally confirmed that they have antiviral, antimutagenic, anti-inflammatory, antibacterial, vasodilatory, and anticancer effects (3). The pronounced ability of flavonoids to complex with ions of transition metals is one of the mechanisms that allow the accumulation of metals in peripheral tissues, which reduces their harmful effect and enhances the defense mechanism against herbivores and pathogenic organisms. This ability was employed for the quantification of flavonoids in various samples, as well as for the detection of trace metals (4).

2. 3-Hydroxyflavone

3-Hydroxyflavone (3HF) is a chemical compound representing the main structure of flavonols, a widely spread subgroup of flavonoids (Figure 1). However, although this compound itself is not found naturally in plants, 3HF can be practical as a model molecule because of its excited-state intramolecular proton transfer (ESIPT) effect (5), in membrane (6) or intermembrane proteins (7) studies. Generally, 3HF derivates prevent the production of free radicals and can therefore be helpful as antioxidant and protective molecule substances (8).

Therefore, the necessity of syntheses of 3HFs in a shorter time period and as high as possible yields has resulted in many innovative methods. The one proposed by Gonduz et al. (9) considers a simple purification and provides the syntheses of 3HFs with a hydroxyl group on the phenyl ring in just one step, which is a significant improvement compared to the current four steps available in the literature, with longer reaction time and a lower yield.
3-Hydroxyflavone is soluble in methanol and ethanol, while its insolubility in water can be improved by encapsulation in cyclodextrins (4). One of the main characteristics of almost all flavonoids is the formation of complexes with metal ions, due to the existence and appropriate layout of one or more ortho hydroxyl phenolic groups, or phenolic groups with a carbonyl group. Since they possess an α-hydroxycarbonyl group, flavonols exhibit a very high affinity to bind metal ions, especially compared to other flavonoids (Figure 2) (10, 11).

Many studies have examined the effects of 3-hydroxyflavone and its structural analogs. One such study dealt with the activation of the pregnane H receptor (PHR), which belongs to a group of nuclear receptors that regulate the expression of genes for many biological processes. It has been shown that 3HF activates this receptor, which may be helpful in the treatment of diseases that depend on the PHR receptor, such as inflammatory bowel disease (12).

It has also been shown that 3HF inhibits the metastasis of human osteosarcoma cells and reduces tumor growth in vivo. This may lead to a clinical trial of osteosarcoma chemotherapy (13).
Like other flavonoids, 3-hydroxyflavone also exhibits antioxidative characteristics. This molecule complexes with metal ions that can induce oxidative stress and is known to suppress the cytotoxicity of lipid peroxides (especially hydroperoxide of linoleic acid) (14).

Adverse effects of 3-hydroxyflavones are manifested when this molecular species is present in increased concentration, which can cause pro-oxidative action, manifested by an increase in the intracellular concentration of free oxygen radicals (ROS) and possible cell damage (15).

Although very similar, the study by Sengupta et al. (16) presented the antioxidant properties of two isomers, 3-hydroxyflavone (3HF) and 7-hydroxyflavone (7HF), against nicotine-associated oxidative stress and injury in cultured renal proximal tubule cells, and correlated their antioxidant properties with their chemical structure. The data elucidated that although both 3HF and 7HF protect renal cells from NIC-associated cytotoxicity, the mechanism of their action is different (16).

Some very inventive applications of 3HF-based compounds are related to carbon monoxide (CO) as an endogenous signaling molecule that influences various biological processes. The therapeutic potential of CO is hindered by its intrinsic toxicity, and its administration therefore carries a possible risk. Photoactivatable CO-releasing molecules (photoCORMs) are an excellent tool to overcome the side effects of untargeted CO administration and provide precise release control. Thus, the study of Russo et al. (17) reported the CO release mechanism of several flavonol derivatives, previously developed as an efficient photoCORM. The study aimed to examine how to enhance the efficiency of CO photorelease from flavonols, and how to minimize photochemical side-reactions, such as self-photooxygenation. Moreover, the study reported the toxicity of tested flavanols on hepatic HepG2 cells in vitro as a significant fact for future possible application.

Intending to find a more comprehensive application of 3HF, researchers have focused on its complexes with metal ions. The syntheses of 3-hydroxyflavone complexes with aluminum and zinc are already reported in the literature (18, 19). In this paper, besides their characterization, the validation of a developed spectrophotometric determination of 3HF based on complexes formed with zinc (Zn-3HF) and aluminum (Al-3HF) is presented. Furthermore, their antioxidant capacity is tested, and more positive issues of 3HF complexes of both tested ions are evaluated.

**Experimental**

### 1. Materials and Instruments

3-Hydroxyflavone, KCl, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) trolox, DPPH (2,2-diphenyl-2-picrylhydrazyl), (Sigma-Aldrich), methanol, acetic acid, CH₃COONa, AlCl₃, ZnCl₂, (Merck), all p.a. purity grade, were used.

The stock solutions of ZnCl₂ (1×10⁻³ molL⁻¹) and AlCl₃ (1×10⁻⁴ mol L⁻¹) were prepared by dissolving ZnCl₂ and AlCl₃ in redistilled water. The stock solution (1×10⁻⁴ mol L⁻¹) of 3HF was prepared by dissolving the appropriate mass in 70% methanol (V/V), and after sonication for 15 minutes, it was stored in a refrigerator.
Acetate buffers (in 70% methanol V/V) with different pH values, previously prepared according to Perrin et al. (20), were used for appropriate spectrophotometric measurements.

Spectrophotometric absorption spectra recording was performed by a *Beckman DU 650 Spectrophotometer* with 1 cm quartz cells. pH-metric measurements were performed by the Thermo Scientific-Orionca with a combined glass electrode (sensitivity ± 0.01 pH).

2. Methods

2.1. Spectrophotometric determination of 3HF

The study of complex formation between 3HF and metal-ions started by testing the dependence of absorbance intensity on pH in acetate buffers (in 70% methanol (V/V)) of different pH values (20). To ensure that quantitative complex formation was achieved, the procedure considered the concentration of metal ions in the mixture was $1 \times 10^{-6}$ mol L$^{-1}$, while the concentration of 3-hydroxyflavone was twenty times higher, $2 \times 10^{-5}$ mol L$^{-1}$.

For calculating the stability constant of the complex, $\beta_2$, modified Bjerrum's method was used (21).

The stoichiometry of the complexation reaction for both complexes was investigated using the equimolar solution variation method (22). Considering the overall equilibrium of metal ion $M^{n+}$ (Zn$^{2+}$ or Al$^{3+}$) and $n$ ligands (L= 3HF), presented as $M^{n+} + nL = [ML_n]^{m+}$, where $n$ can be determined from the plot of the absorbance as a function of the mole fraction, $x$, of the added ligand, in the maximum, $n$ is

$$n = \frac{x_{\text{max}}}{1-x_{\text{max}}}$$

(1)

The calibration curve method was used for spectrophotometric determination of 3HF, requiring prepared solutions containing a constant concentration of ZnCl$_2$ or AlCl$_3$ and different concentrations of 3HF in acetate buffer (in 70% methanol (V/V)) at an appropriate pH (Zn-3HF at 7.99 and Al-3HF at 4.90). The series of seven standard solutions in the range $1 \times 10^{-6} - 2 \times 10^{-5}$ mol L$^{-1}$, in the presence of the excess of Zn(II) ion or Al(III) ion, $3 \times 10^{-5}$ mol L$^{-1}$, were prepared. The blank was acetate buffer in 70% methanol (V/V) 7.99/4.90.

The obtained data were used to calculate analytical validation parameters for spectrophotometric methods for both complexes according to relevant literature (23, 24).

The limit of detection (LOD) was calculated from the equation:

$$\text{LOD} = 3.3 \frac{Sb}{a}$$

(2)

where: $Sb$ – standard deviation in intercept; $a$ – slope of the calibration line, while the following relation was used to calculate the limit of quantification (LOQ):

$$\text{LOQ} = 10 \frac{Sb}{a}$$

(3)
2.2. Antioxidative tests

Experimental measures and conditions for testing the antioxidative ability of 3HF and corresponding zinc and aluminium complexes were performed according to the procedures for the DPPH tests previously reported for chosen flavonoids (25-27), with the solutions of 3HF at a concentration of $5 \times 10^{-6}$ mol L$^{-1}$ and concentrations of Zn$^{2+}$ and Al$^{3+}$ ion c = $2.5 \times 10^{-6}$ mol L$^{-1}$.

Furthermore, 3HF and its complexes were tested for scavenging free radicals potential by the stable radical reagent 1,1-diphenyl-2-picrylhydrazyl, DPPH. The hydrogen atom or electron donation abilities of the tested complexes and pure 3HF were estimated based on the bleaching of the methanol solution of DPPH, according to previously reported procedures (25, 26). In brief, 1 mL of the tested samples was added to 4 mL of 0.004 % methanol solution of DPPH. After a 30-minute incubation period at room temperature, protected from light, the absorbance was read against a blank at 517 nm. The inhibition of free radicals by DPPH in percentages (% INH) was calculated from the equation (4):

$$INH = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100\%$$

In the formula (4), $A_{\text{control}}$ is the absorbance of the solution containing all reagents except the test compound, while $A_{\text{sample}}$ is the absorbance of the prepared tested complex or 3HF.

The samples were also checked for their ability to scavenge ABTS free radicals (ABTS$^+$). The stock solution of ABTS$^+$ was prepared as proposed in the literature (28). In brief, according to Pavun (27), the test sample of 3HF was dissolved in 5 mL of ABTS$^+$ solution, mixed thoroughly, diluted to 10 mL with PBS buffer, and incubated in the dark at room temperature. After 3 minutes, the absorbance was read at 734 nm with a PBS buffer as blank. The results were expressed as % INH (percentage of ABTS$^+$ inhibition) according to the equation:

$$\% \ INH = \frac{[A_{\text{ABTS}^+} - A_{\text{sample}}]}{A_{\text{ABTS}^+}} \times 100$$

where $A_{\text{ABTS}^+}$ is the absorbance of ABTS$^+$ solution + 5 mL of PBS buffer, and $A_{\text{sample}}$ is the absorbance of the solution in the presence of the sample after 3 min of reaction.

Results

1. Complex formation between 3HF and metal-ion

The dependence of absorbance of the metal-ions complexes of 3HF intensity on pH was examined. Figure 3 represents the absorbance as a function of the pH solution, where a strong pH dependence can be noticed. The maximum absorbance of the Zn–3HF complex is $\lambda_{\text{max}} = 400$ nm, at pH 7.85 (Figure 3a). The maximum absorbance of the Al–3HF complex is $\lambda_{\text{max}} = 343$ nm, at pH 4.91 (Figure 3b). The solutions of ZnCl$_2$ and AlCl$_3$ do not exhibit significant absorbance in the range of 250–500 nm.
Using an equimolar solution variation, it was determined that 3HF makes a complex with Zn(II) ion at pH 7.98, in the stoichiometric ratio 3HF : Zn$^{2+}$ ion = 2 : 1, with an absorption maximum on $\lambda_{\text{max}} = 400$ nm (Figure 4a). Al-3HF complex was formed in the stoichiometric relation 3HF : Al$^{3+}$ ion = 2 : 1 as well, but at pH 4.91, with an absorption maximum on $\lambda_{\text{max}} = 343$ nm (Figure 4b). The compositions of these complexes were also checked by the mole ratio method, confirming the 3HF and M$^{m+}$ (Zn$^{2+}$ or Al$^{3+}$) ratio is 2 : 1 for both complexes formed at an appropriate pH against 70% methanol (V/V) as a blank.
2. Spectrophotometric method development

The high values of the stability constants of complexes Zn-3HF (log $\beta_2 = 14.35$ at pH =7.98) and Al–3HF (log $\beta_2 = 22.63$ at pH =4.91) allow the quantification of 3HF established on these complexes. The construction of the calibration curve, $A = f(c_{3HF})$ was the same for both metal ions.

For the Zn-3HF complex, the series of solutions were prepared at pH 7.98 in 70% methanol (V/V), and the absorbance was measured at $\lambda = 400$ nm.

Good accuracy and reproducibility of the method are reflected in a high correlation coefficient $R= 0.9892$, with LOD and LOQ calculated as $3.82 \times 10^{-7}$ mol L$^{-1}$ and $1.15 \times 10^{-6}$ mol L$^{-1}$.

In the case of the Al-3HF complex, the solutions were prepared with 70% methanol (V/V), with pH 4.91, while the absorbance was measured at $\lambda = 343$ nm. Good linearity of the calibration curve and small scatter of experimental points resulted in a higher correlation coefficient, $R = 0.99986$. The LOD was $1.83 \times 10^{-7}$ mol L$^{-1}$, while the LOQ was $5.50 \times 10^{-7}$ mol L$^{-1}$.

The accuracy of both developed variations was tested for three different concentrations of 3HF in the range of 2-10 µmol L$^{-1}$, with five repeated measurements for each. The accuracy and repeatability of the method are reflected in very good recovery and a low coefficient of variation (CV). The obtained analytical parameters are presented in Table I.

<table>
<thead>
<tr>
<th>pH</th>
<th>Method based on Zn-3HF</th>
<th>Method based on Al-3HF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.98</td>
<td>4.91</td>
</tr>
<tr>
<td>Wavelength</td>
<td>400 nm</td>
<td>343 nm</td>
</tr>
<tr>
<td>Stability</td>
<td>log $\beta_2 = 14.35$ at pH =7.98</td>
<td>log $\beta_2 = 22.71$ at pH =4.90</td>
</tr>
<tr>
<td>Molar absorption coefficient</td>
<td>$3.04 \times 10^4$ cm$^{-1}$ mol$^{-1}$ dm$^3$</td>
<td>$3.94 \times 10^5$ cm$^{-1}$ mol$^{-1}$ dm$^3$</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$A = (4.32 \pm 0.05) \times 10^3 \cdot c - (0.0022 \pm 0.0005)$</td>
<td>$A = (1.82 \pm 0.01) \times 10^4 \cdot c + (0.005 \pm 0.001)$</td>
</tr>
<tr>
<td>Linearity range</td>
<td>$1 \times 10^6$ mol L$^{-1}$ - $2 \times 10^5$ mol L$^{-1}$</td>
<td>$1 \times 10^6$ mol L$^{-1}$ - $2 \times 10^5$ mol L$^{-1}$</td>
</tr>
<tr>
<td>LOD</td>
<td>$3.82 \times 10^{-7}$ mol L$^{-1}$</td>
<td>$1.83 \times 10^{-7}$ mol L$^{-1}$</td>
</tr>
<tr>
<td>LOQ</td>
<td>$1.15 \times 10^6$ mol L$^{-1}$</td>
<td>$5.50 \times 10^7$ mol L$^{-1}$</td>
</tr>
<tr>
<td>Recovery</td>
<td>100.63 %</td>
<td>100.17 %</td>
</tr>
<tr>
<td>CV</td>
<td>0.27-0.93 %</td>
<td>0.25-1.00 %</td>
</tr>
</tbody>
</table>
According to the presented data, it can be concluded that a certain advantage can be given to the variation of the spectrophotometric quantification of 3HF developed on its reaction with aluminium. The resulting Al-3HF complex is more stable than Zn-3HF, so the LOD and LOQ values are expected to be lower. In addition, an advantage of this determination is that it is performed in an acidic medium in contrast to a slight base, which is always favored in real samples.

Interestingly, the Zn-3HF complex was previously employed for the spectrofluorimetric determination of zinc traces in tap water, multivitamin tablets/capsules, and hair shampoo, with LOD for Zn\(^{2+}\) at 1.5 ppb (29). Herein we used the same complex for 3HF determination, applying simple and low-cost spectrophotometry, and obtained remarkably low values of LOD for 3HF.

As previously shown (25-27), the selectivity of the developed spectrophotometric method concerning the presence of some other flavonoids, including morin, hesperidin, quercetin, or rutin, is ensured by the choice of pH and the wavelength at which the recording is performed.

3. Antioxidative ability

As it is well known, numerous tests can be used to define the antioxidative ability of pure compounds or naturally sourced extracts (30). Which one will be applied depends on many external factors such as pH, solvent, and system characteristics. There is therefore no universal method or universal parameter based on which all compounds could be absolutely compared; it depends on the reaction type being tested and on the mechanism, but this can be overcome by combining several tests.

The spectrophotometric methods used in this study are fast, easy, and widely available, but with somewhat conflicting results due to the influence of some of the factors mentioned above. The results of performed antioxidative tests for 3HF and its complexes are represented in Table II.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% INH DPPH (30 min)</th>
<th>% INH ABTS (3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3HF</td>
<td>58.9</td>
<td>67.6</td>
</tr>
<tr>
<td>Zn-3HF</td>
<td>70.2</td>
<td>77.1</td>
</tr>
<tr>
<td>Al-3HF</td>
<td>55.3</td>
<td>59.4</td>
</tr>
</tbody>
</table>

Both applied tests showed that the complexation of 3HF with Zn\(^{2+}\) significantly enhances the antioxidant ability. As reported in our previous works, the complexation with Zn\(^{2+}\) does not necessarily positively contribute to the flavonoids' antioxidative potential (24-27). ABTS and FRAP tests performed for the complexes of quercetin, morin
and rutin with zinc exhibited about the same or slightly higher antioxidative capacities than pristine flavonoids. In contrast, in the DPPH test there was an increase in activity when zinc was binding with hesperidin and quercetin. None of the three tests recorded the antioxidative activity of the solution of Zn$^{2+}$ itself.

As for the Al-3HF complex, the antioxidant activity (DPPH assay) was slightly lower, but still not significantly different compared to 3HF. The fact that zinc electrode potential is less positive than aluminium might explain such a finding. The results of the ABTS assay showed that the Al-3HF complex is less active than pristine 3HF. This finding agrees with the literature data reported for the complex of Al$^{3+}$ with luteolin (31).

Another direction for future research could be based on the already established effect of 3HF as a safe chelating reagent of cobalt ions, but with low stability of the formed complex (Co-3HF) over time (32). 3HF is not considered a toxic compound (3HF alone caused a significant toxic effect on cells at high concentrations of $1 \times 10^{-3}$ mol L$^{-1}$). The stability constants of Zn-3HF, and especially Al-3HF, are considerably higher than those of Co-3HF (32). It can be expected that 3HF can be a candidate for future testing as a chelating reagent for zinc or aluminium, especially having in mind the toxicity of aluminium ions, giving this study additional importance.

**Conclusion**

Because 3-hydroxyflavone forms stable complexes with Zn$^{2+}$ and Al$^{3+}$ ions, very simple, fast, low-cost spectrophotometric methods for determining 3HF have been developed and validated. The advantage of this eco-friendly method, in addition to its availability, time, and price performance, is reflected in the fact that no toxic solvents are used for the mobile phase, as they often are for HPLC.

A slight advantage could be given to the version with an Al$^{3+}$ ion due to very low LOD and LOQ values and acidic working pH.

At the same time, the significant antioxidant capacity of the Zn$^{2+}$ ion complex of 3HF, confirmed by DPPH and ABTS tests, makes this complex a promising candidate for future investigations in this field. In any case, further experiments for stability and efficiency evaluation of the respective complexes in different environments must not be forgotten.

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Uporedno spektrofotometrijsko određivanje 3-hidroksiflavona bazirano na kompleksima cinka i aluminijuma i njihovi antioksidatni profili

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Kratak sadržaj

Flavonoidi, jedinjenja biljnog porekla, vekovima su bili veoma važne aktivne komponente u tradicionalnoj medicini. Veliki broj njihovih potencijalnih ili već potvrđenih efekata uključuje antivirusna, antimutagena, antiinflamatorna, antibakterijska, vazodilatatorna i antikancerogena svojstva. Promovisanje biljne ishrane, uz isticanje koristi konzumiranja flavonoida, u današnje vreme postalo je sve privlačnije. 3-Hidroksiflavon (3HF) je strukturni stub svih flavonola, važne klase flavonoida. Iako sam 3HF ne postoji u biljkama per se, on ispoljava mnoge svoje efekte zahvaljujući osobini da sprečava stvaranje slobodnih radikala. Ovaj rad je fokusiran na karakterizaciju kompleksa 3HF sa jonima cinka(II) i aluminijuma(III) (Zn-3HF i Al-3HF, respektivno). Izvršena je karakterizacija ovih kompleksa i razvijena brza i pristupačna metoda za spektrofotometrijsko određivanje 3HF, na osnovu formiranja kompleksa Zn-3HF i Al-3HF, sa veoma niskim vrednostima LOD i LOQ. Mala prednost je data modifikaciji sa Al³⁺ na pH 4,91 zbog izuzetno niskih vrednosti LOD i LOQ, 1,83×10⁻⁷ mol L⁻¹, odnosno 5,50×10⁻⁷ mol L⁻¹, kao i visokog koeficijenta korelacije, R=0,99986. Pored toga, antioksidativni kapaciteti sintetizovanih kompleksa Zn-3HF i Al-3HF, kao i samog 3HF, ispitani su DPPH i ABTS testovima i doveli su Zn-3HF kompleks u prvi plan za dalja ispitivanja kao potencijalnog antioksidativnog agensa.

Ključne reči: spektrofotometrija, 3-hidroksiflavon, cink kompleks, aluminijum kompleks, antioksidativni kapacitet