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Spectrophotometric determination of morin in strawberries and their antioxidant activity

Leposava Pavun[1], Aleksandra Janošević-Ležaić[1], Snežana Uskoković-Marković[2,*]

[1]University of Belgrade - Faculty of Pharmacy, Department of Physical Chemistry and Instrumental Methods, Vojvode Stepe 450, 11221 Belgrade, Serbia

[2]University of Belgrade - Faculty of Pharmacy, Department of Analytical Chemistry, Vojvode Stepe 450, 11221 Belgrade, Serbia

[*]Corresponding author: Snežana Uskoković-Marković,
E-mail: snezaum@pharmacy.bg.ac.rs

Abstract

Morin is one of the flavonoids with intensive antioxidant activity. With the aim to use its benefits on human health, there is an increasing trend to pay attention to its content in food or supplements. The simplicity and low cost of spectrophotometric determination based on the formation of a morin complex with Zn^{2+} ion (stoichiometric ratio 1 : 1), at pH 7.98 and 392 nm, give it an advantage over other methods that can be used for morin quantification. The concentration range over which the response was linear was 0.151 – 4.533 mg L^{-1}. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.030 mg L^{-1} and 0.091 mg L^{-1}, respectively. The developed method was successfully applied for the determination of the morin content in strawberries. Additionally, the antioxidative abilities of strawberry extracts and morin, determined by DPPH and FRAP tests, were compared and discussed.

Key words: morin; strawberries; spectrophotometry; zinc complex.

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Introduction

Flavonoids, the biggest group of plant polyphenol compounds, as secondary cell metabolites, are pigments of great importance for plants. The color of flowers and fruit plays a very important role in attracting insects and bees in order to pollinate and spread the seeds (1). Flavonoids have a characteristic absorption spectrum, which is why even when the human eye does not notice them as the color of flower petals, they are visible to insects and direct them to the central part of the flower where nectar and pollen are located (2). Flavonoid aglycones in the form of exudates are often found on the surface of leaves and flowers in the form of a powder coating mixed with the cuticle, so it is considered that such localization protects the plant from UV radiation (3). Flavonoids are also produced against biotic stress, such as in the case when plants are exposed to pathogen invasion (4).

Flavonoids possess synergistic effects in antioxidant activity, so it is supposed that these are the most useful phytochemicals found in food. Due to that fact, these compounds have a positive effect on human health, although they are not essential nutrients (5). The antioxidant activity of flavonoids is attributed to their involvement in alleviating or preventing cellular oxidative stress. Oxidative stress is caused by the overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH•) and superoxide (O_2^-•) (6). Radicals can be generated either enzymatically, for example, xanthine / xanthine oxidase or reductase, or non-enzymatically, by means of a transition metal alone or in combination of a transition metal with a reducing agent such as ascorbate. In both cases, flavonoids can interfere not only with free radical propagation reactions, but also with radical formation, either by chelating the transition metal or by inhibiting the enzymes involved in the initiation reaction (6, 7).

As it is well-known that conditions such as aging, cancer, cardiovascular, and neurodegenerative diseases are associated with cellular oxidative damage, flavonoids are reasonably pointed to as very important nutraceuticals in the human diet. In recent years, flavonoids have been extremely interesting for researchers, and consequently there are numerous supplements on the world market that are based on the activities of this group of compounds (8).

The subgroups of flavonoids are: flavones, flavonols, isoflavonoids, flavanones, anthocyanins and chalcones (9). The most studied flavonols, with confirmed beneficial effects on human health, are quercetin, kaempferol, myricetin and rutin (9).

The flavonol morin (2', 3, 4', 5,7-pentahydroxyflavone) is a bioactive flavonoid found in yellow Brazilian wood, figs, and other plants of the Moraceae family, such as mulberry, and some of the historical Chinese medicinal plants (10). Morin is present in vegetables such as kale, broccoli, lettuce, and tomatoes, and is less common in fruit such as apples, strawberries, grapes, and berries (11). Beneficial effects of berries, due to high flavonoids content, especially attract scientists' attention (12).

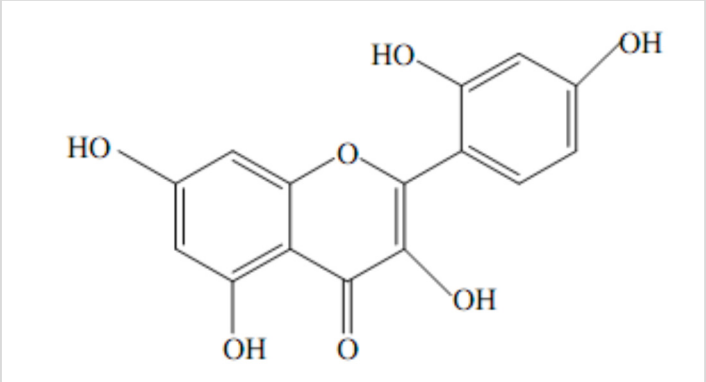

Figure 1. Morin structure
Slika 1. Struktura morina

Morin possesses various bioactivities, including antiviral, anti-inflammatory, antitumor and cardioprotective activities (13-16). Morin hydrate is amphiphilic - free hydroxyl groups on both rings increase its solubility in organic solvents. This property can facilitate the uptake of morin hydrate across the cell membrane, which contains both hydrophilic proteins and hydrophobic lipids (17).

Morin is one of the antioxidants that can protect various human cells, such as myocytes, endothelial cells and hepatocytes against oxidative radicals formed in situ (18). Further, the results indicate that morin can protect cardiovascular and brain tissue by reducing oxidative stress, inflammation, and apoptosis (19).

As an interesting but not unexpected application, morin has been recommended as a food preservative, such as in the case of delaying the senescence of banana (20). Morin has also shown significant protective effects against UV radiation, so its introduction into a topical formulation can be beneficial for skin health (21).

Evaluating numerous potential bioactivities of morin depended on finding the appropriate methods for its determination in food, extracts, pharmaceutical formulations and biological fluids. High Performance Liquid Chromatography (HPLC) is a widely used method to successfully separate and analyse flavonoids, including morin (22, 23). Up to now, the analysis of morin has been also accomplished by spectrofluorimetry, with very low limits of detection and quantification (24). Such methods could be applicable in following the level of morin in biological samples, during the studies performed with the aim of confirming the potential relation between some specific diet and health effects. The disadvantages of both methods for routine analysis are moderate (for HPLC) or high costs (for spectrofuorimetry), and not so available instrumentation.

The ability of flavonoids to form complexes with metal ions, and the structural characteristics of flavonoids that allow the absorption of light in the visible part of the spectrum, were used for the application of spectrophotometry as a method in their determination in different samples. The aim of this study was to develop a simple and

efficient spectrophotometric determination of morin based on its complex formation with zinc (II) ion, and to employ the method in the morin quantification in strawberries. Complementary tests of antioxidant ability of the same sample were also tested.

Experimental

Materials and Methods

Reagents and Materials

Morin, KCl (*Fluka AG*), trolox, 2,2-diphenyl-2-picrylhydrazyl - DPPH, 2,4,6-tris (2-pyridyl)-s-triazine - TPTZ *(Sigma-Aldrich),* methanol, CH_3COOH, CH_3COONa, zinc-chloride, (*Merck*), $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$ (*Zorka-Šabac*), all p.a. purity grade, were used without further purification.

The stock solution of zinc-chloride (1.0×10^{-3} $molL^{-1}$) was prepared by dissolving $ZnCl_2$ in doubly distilled water. The stock solution of morin (1.0×10^{-4} $molL^{-1}$) was prepared by dissolving morin in methanol (70 % v/v), with sonification in an ultrasound bath for 15 min, and was stored in a refrigerator.

Working solutions of the zinc (II)-morin complex were prepared by dilution of the stock solutions of zinc (II) (2.5×10^{-5} $molL^{-1}$ $ZnCl_2$) and morin (5.0×10^{-7} to 1.0×10^{-5} $molL^{-1}$). Acetate buffers, previously prepared according to the literature (25), were used for all spectrophotometric measurements.

Instruments

The *Beckman DU 650* Spectrophotometer, with 1 cm quartz cuvettes, was used for the absorption spectra recording, defining the complex composition, and determination of morin content in the test samples.

An MA 5703 Iskra pH meter, Slovenia was used for buffers preparation, with Rusell combined pH electrode (sensitivity ± 0.01 pH units), in the range of 0 - 14 pH. A Bendelin Sonorex Super ultrasonic bath, Model RK 512K, was used in the preparation of the solutions. For the sample preparation, according to the procedure, a Tehtnica LC-320centrifuge, with 6000 rpm and a Gorenje blender were used.

The quantitative determination of morin

Spectrophotometric determination of morin – calibration curve

The calibration curve method was used, requiring prepared solutions containing constant concentration of $ZnCl_2$ and different concentrations of morin in acetate buffer (in 70 wt% methanol) pH 7.98. The blank was acetate buffer in 70% methanol pH 7.98. Morin is sparingly soluble in water and soluble in methanol; 70% V / V methanol proved to be the most favorable solvent for the formation of the Zn^{2+} - morin complex.

The obtained data were used to calculate analytical validation parameters for the spectrophotometric method according to the literature (26, 27). The limit of detection (LOD) was calculated by establishing the minimum level at which morin can be detected, according to the following formula:

$$LOD = 3.3\ Sb/a \qquad (1)$$

where: *Sb* – standard deviation in intercept; *a* – slope of calibration line

The limit of quantification (LOQ) was determined using the following formula:

$$LOQ = 10\ Sb/a \qquad (2)$$

The determination of morin content was performed indirectly by measuring the absorbance of the morin-zinc complex at 392 nm, and by applying the calibration curve method. A series of standard solutions of morin and zinc (II) - ions was prepared. In these solutions, the concentration of morin was varied, while the concentration of zinc (II) ions was constant. Zinc (II) ions were present in large excess in order for the reaction of zinc (II) - ions and morin to be maximally shifted in the direction of complex formation and for morin from standard solutions to be quantitatively complexed. The acetate buffer used is the pH value for which the zinc (II) - morin complex has been shown to have maximum stability and maximum absorption.

Preparation of strawberry extract

A few strawberries were chopped in a blender, and then 10 grams were weighted on an analytical balance and transferred quantitatively to a suitable container. After that, 10 mL of 70% methanol were added and shook vigorously to perform morin extraction. The dish was closed and left in a dark place for 12-24 hours. The obtained mixture was mixed on a magnetic stirrer for 15 minutes at 900 rpm, and then the extract was filtered through a Millipore Membrane Filter, pore size 0.45 μm. The resulting extract - marked as solution A, was stored at 4 ° C in a dark place.

Antioxidative activity

DPPH test

To determine the antioxidative activity of morin, it was necessary to construct the appropriate calibration curve, starting from 100 mg L^{-1} trolox solution and 40 mg L^{-1} solution of DPPH (28). The appropriate volume of already prepared trolox solution was added to 12.5 mL of DPPH in six 25 mL volumetric flasks, to obtain the final concentration of trolox 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}, and filled to the mark with methanol. As the control, 12.5 mL of DPPH solution was diluted with methanol in a 25 mL volumetric flask. The absorbance was measured at λ=514 nm, after 60 min,

with absolute methanol as blank. The antioxidant activity of the strawberry extract was tested by mixing 5 mL of an already prepared extract, 12.5 mL of the DPPH working solution into a 25 mL volumetric flask and diluting by methanol.

The inhibition of the DPPH free radical was calculated according to Equation 3:

$$I = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\% \qquad (3)$$

where: $A_{control}$ – absorbance of the control mixture; A_{sample} – absorbance of the standard or prepared sample.

FRAP test

A slightly modified FRAP test can also be employed for the determination of antioxidative activity (29). The FRAP reagent for the calibration curve was prepared by mixing acetate buffer (300 mmol L^{-1}, pH=3.6), TPTZ reagent (10 mmol L^{-1} in 40 mmol L^{-1} HCl) and $FeCl_3 \cdot 6H_2O$ (20 mmol L^{-1}) in volume ratio 10:1:1. Five solutions were prepared starting with 3 mL of FRAP reagent, and 0.1 mL of $FeSO_4$ in the range 0.2-1.0 mmol L^{-1}. Absorbance was read at 593 nm, against the blank (3 mL FRAP reagent and 0.1 mL water). The calibration curve was constructed as the read absorbance in the function of $FeSO_4$ concentration.

Results and Disscusion

Characterization of morin complex with zinc (II)-ion

Our one-decade long experience focused on the abilities of flavonoids to form complexes with transition metals, and results were reported for developed spectrophotometric (30-32) and spectrofluorimetric (24, 33-36) methods for the determination of flavonoids in different samples. Previously we have learned that aluminum and zinc could be particularly useful for that purpose, but it is challenging to find the optimal conditions of the proposed determination method.

Interestingly, morin is especially important as a complexing reagent for spectrofluorimetric determination of aluminum. The results of studies performed in the early twentieth century have shown that the accumulation of aluminum in the body can lead to pathological conditions (37). Due to the potentially negative pharmacological effects of aluminum, it is important to monitor its level in environmental and biological samples. For this purpose, spectrofluorimetric determination of aluminum, based on the formation of a complex with morin, proved to be an ideal method, with very low LOD and LOQ (38).

Morin is known as an easily interacting flavonol with metal ions, forming the complexes defined as MMor and $MMor_2$ (39). Depending on the medium acidity, morin can be found in seven forms: protonated (H_6R^+), molecular (H_5R), as well as deprotonated

(H_4R^-, H_3R^{2-}, H_2R^{3-}, HR^{4-}, and R^{5-}) (40). The most acidic proton in the morin structure is 3-OH, with pKa=3.46, which exhibits a greater ability to complex compared to 5-OH. The mentioned characteristics are promising as the basis for the zinc complex based spectrophotometric method for the determination of morin. In the beginning, it is necessary to find the conditions, such as pH where the complex is the most stable, and the wavelength of the complex absorption maximum.

Absorption spectra of the complex

Upon reaction in methanolic solution morin and zinc (II)-ion form a yellow- orange complex in the pH range 2.31 – 9.45. A series of acetate buffers was prepared in the pH range 2.31 – 9.45, with 70% V/V methanol as a solvent. The absorption spectra were recorded using the solutions 2.5×10^{-6} mol L^{-1} of $ZnCl_2$ and 5×10^{-5} mol L^{-1} morin and their mixture, where the concentrations of components were the same as in the single solutions, at a constant pH. The absorption spectra were recorded using 70 % methanol as a blank. Also, the calculated spectrum of the complex, $A_{compl} = f(\lambda)$, was obtained using the following equation for the calculation of the complex absorbance, A_{compl}:

$$A_{compl} = A_{\text{mix}} - A_{\text{Zn}} - A_{\text{mor}}$$

where A_{mor}, A_{Zn} and A_{mix} are the absorbances of the solutions of morin, $ZnCl_2$ and their mixture, respectively, at the corresponding wavelengths.

Absorption spectra of the Zn-morin complex in methanolic solution in different pH values are shown in Figure 2. There is an isosbestic point at 368 nm.

The absorption maximum of this complex in a strongly acidic medium is 355 nm, and with increasing pH the solution shifts batochromically, and in the base medium the maximum absorbance is settled at 392 nm.

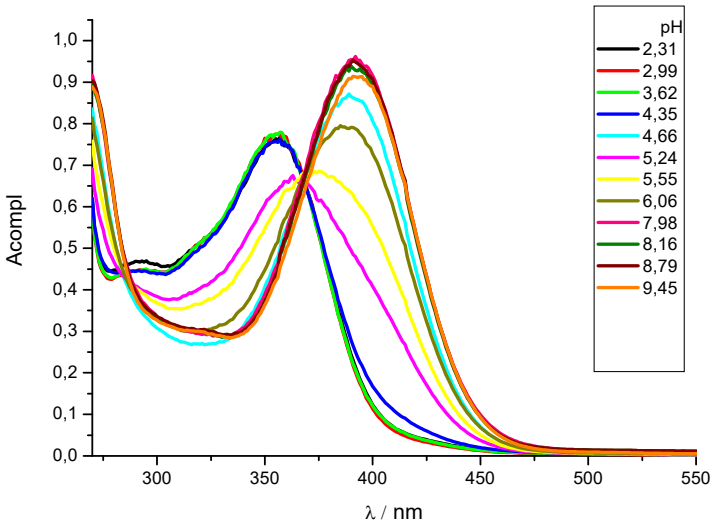

Figure 2. Absorption spectra of a mixture of morin and zinc (II) - ions at different pH
Slika 2. Apsorpcioni spektri smeša morina i cink(II) – jona na različitim pH

To examine the dependence of absorbance of the complex on pH, the measurements were made in acetate buffers of different pH values, prepared according to Perrin (25). By subtracting the relevant absorbances of the $ZnCl_2$ and morin solutions from their mixture, the curve A = f (pH) was obtained for 392 nm (Figure 3).

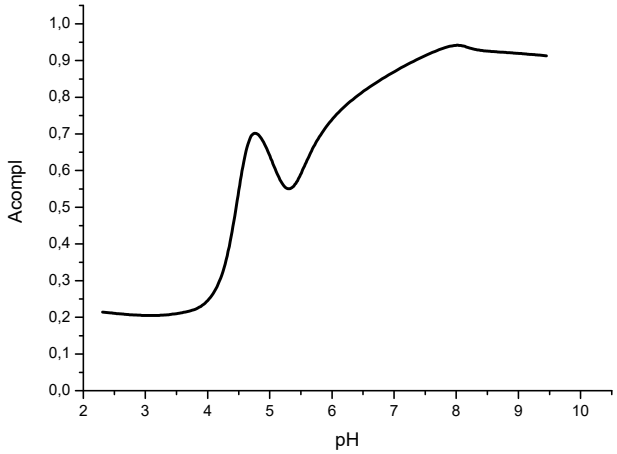

Figure 3. Dependence of the absorbance of the Zn^{2+}-morin complex at 392 nm on the pH of the solution
Slika 3. Zavisnost apsorbancije Zn^{2+}-morin kompleksa na 392 nm od pH vrednosti rastvora

The stoichiometry and the stability constant of the complex

The stoichiometry of the reaction of complexation was investigated by using Job (41) and molar ratio (42) methods. Considering overall equilibrium of Zn^{2+} and *n* ligands (L), presented as $Zn^{2+} + nL = [ZnL_n]^{2+}$, where *n* can be determined from the plot of the absorbency as a function of the mole fraction, *x,* of the added ligand. In the maximum, n is

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

The composition of the complex was determined by the method of variation of equimolar solutions (Job method). The absorbances of the series of solutions formed by mixing equimolar solutions of $ZnCl_2$ and morin (5×10^{-5} mol L^{-1}) at different ratios and a constant value of pH 7.98 were measured at 392 nm, *i.e.*, the Job's method was employed. The blank was a solution of $ZnCl_2$ with the same concentration and pH as in the employed mixture. A typical plot according to Job method is presented in Fig. 4.

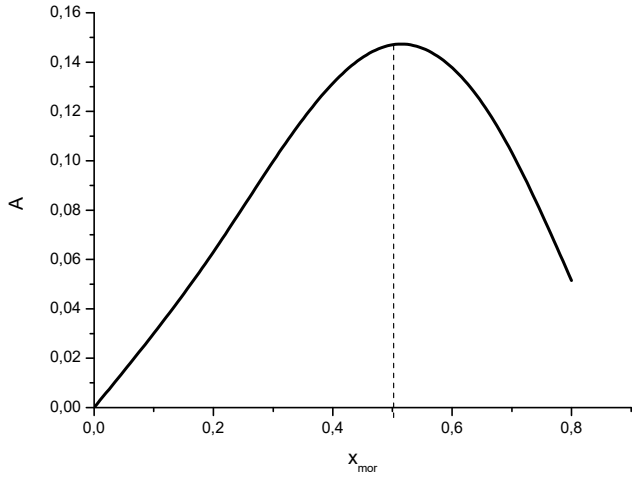

Figure 4. Determination of the composition of Zn^{2+}-morin complexes by the method of equimolar solutions

Slika 4. Određivanje sastava Zn^{2+}-morin kopleksa metodom ekvimolarnih odnosa

At pH 7.98 the stoichiometry is *n* = 1. The composition of zinc (II) to morin was also estimated by the mole ratio method. The result confirms the zinc (II) – morin ratio 1:1 for the complex formed at pH 7.98.

$$K_d = 3.55 \times 10^{-6} \text{ mol } L^{-1}.$$

T

Data from the zinc (II) - morin complex dependence curve for absorbance values at different pH values and using equations derived from the complex formation reaction and morin dissociation constant, the general concentration constants of the zinc (II) - morin complex stability, β_1 at different pH values were calculated. The results are presented in Table I.

Table I Values of general stability constants of zinc (II) - morin complex (β_1)

Tabela I Vrednosti opšte konstante stabilnosti kompleksa cink(II) - morin (β_1)

pH	4.76	6.43	7.49	8.00	8.16	8.39
β_1 log	5.56	5.14	5.87	7.12	6.24	6.01

The stability of the zinc (II) - morin complex was found to be highest at pH 8.00. The obtained values of general concentration constants of stability of the zinc (II) - morin complex are relatively high, which means that a stable complex is formed under the given conditions, so it is possible to quantitatively determine the morin content based on this complex.

Spectrophotometric determination of morin

Method development

The selectivity of spectrophotometric determination of morin based on its complex with zinc (II) ions, regarding similar flavonoids able to react with zinc ions, is achieved by carefully chosen conditions of the determination procedures, such as pH and the wavelength.

The calibration curve for the absorption in the function of the morin concentration was constructed, for the series of seven standard solutions in the range 5×10^{-7} – 1.5×10^{-5} mol L^{-1} (i.e. 0.151 – 4.533 mg L^{-1}), in the presence of an excess of zinc (II) ion, 3×10^{-5} mol L^{-1} . Solutions were prepared with methanol 70% V/V, with pH 7.98, and the absorbance was measured at λ= 392 nm. The calibration curve was defined by the following equation:

$$A = (1.64 \pm 0.05) \times 10^{4} \cdot c - (0.0050 \pm 0.0005)$$

Good accuracy and reproducibility of the method are reflected in a high correlation coefficient R= 0.9959.

The limit of detection (LOD) and the limit of quantification (LOQ) were 1.01×10^{-7} mol L^{-1} (i.e., 0.030 mg L^{-1}) and 3.05×10^{-7} mol L^{-1} (i.e., 0.091 mg L^{-1}), respectively.

Morin content in strawberries

To determine the content of morin, it was necessary to prepare 25 mL of solution B, adjusting the volume of the previously prepared strawberry extract - solution A, so that morin concentration in the solution B was in the linearity range of the calibration curve, A = f (c_{morin}). After adding 0.3 mL of zinc(II) stock solution, the absorbance of the solution was measured at λ= 392 nm, and the concentration of morin calculated according to equation. From the obtained concentration of morin c = 2.76×10^{-4} g L^{-1}, the content of morin can be recalculated and expressed to 100 g of strawberries. The applicability of the determination procedure is confirmed by the standard addition method in our preliminary experiments.

The obtained results of 0.28 mg morin/100 g of strawberries are comparable to literature data [12], and surely obtained with a simpler and lower cost method, as well.

Antioxidative activity

DPPH test

DPPH test *(Scavenging of 2,2-Diphenyl-1-picrylhydrazyl Radical Assay)* is probably the most commonly used test for the determination of antioxidative ability of natural products, based on the H-atoms or electrons exchanging between antioxidant and DPPH radicals in the solution. The colour changing is spectrophotometrically followed at *λ*=514 *nm*.

The stern approach of DPPH determines the reaction, because small molecules access this radical more easily and have a relatively higher antioxidant power. On the other hand, very large antioxidants, which react quickly with the peroxyl radical, in this case react slowly or do not react. The DPPH reaction is "temporal" and can last from 20 min to 6 h.

For the DPPH test, using a series of standard solutions, the following equation for the calibration curve was obtained: RSC = 13.654·c – 1.1307, where *RSC* is the neutralization level of *DPPH*, and C is a trolox concentration (*mg/L*). The recovery value for this determination was *R*=0.9933.

Strawberry extracts were treated in the same way as standard morin solutions in the DPPH test. Using the already constructed standard curve for this test, the absorbance of A = 0.2122 for strawberry extract was obtained, corresponding to the degree of neutralization of DPPH reagent as RSC = 50.88%, or trolox concentration of 3.81 mg L^{-1}.

As a control, DPPH solution was used without the addition of any extract, which corresponds to RSC = 0%, while 70% methanol was used as a blank, and measurements were performed at λ = 514 nm.

FRAP test

FRAP test (Ferric Ion Reducing Antioxidant Power Assay) was originally developed for the determination of the content of reducing substances in the blood plasma, but later found an application for the determination of the antioxidant capacity of plant extracts. Electron donation by the antioxidant reduces the ferri-tripyridyltriazine [Fe^{3+}-TPTZ] complex to an intense blue ferro-tripyridyltriazine [Fe^{2+}-TPTZ] complex at low pH values. The reaction was monitored spectrophotometrically at 593 nm (maximum absorption of the reduction product). To determine the antioxidant activity of the obtained strawberry extract, the FRAP test according to the Benzie Strain method with certain modifications was used.

As a result, *FRAP* test gave the following equation for the calibration curve: $A = 0.8268 \cdot c + 0.0096$, where A was absorbance, and C the concentration of Fe^{2+}, while the recovery value was R=0.9996.

Strawberry extracts were treated in the same way as standard morin solutions in the FRAP test. Using the already constructed standard curve for this test, strawberry extract showed A = 0.5691, which is equivalent to an iron (II)-ion concentration of $c = 0.68$ mmol L^{-1}.

The antioxidative activity of fruit and vegetables with considerable flavonoids content is well-known, but it is always necessary to test this activity using several tests, with the aim of getting information about the nature of antioxidative profile (43). Besides flavonoids, other polyphenols and vitamin C also contribute significantly to the antioxidative potential of plant samples (44).

Besides the antioxidative effects of morin, the bioactivity of the zinc-morin complex is very promising itself (45). That interesting fact gives new insight into multicomponent supplements, opening new topics for investigation, such as possible interactions of ingredients, methods of quantification and others.

Conclusion

The ability of morin to complex with zinc (II) ion is successfully used for developing a simple, low-cost and accurate spectrophotometric determination of morin in real samples, such as strawberries. The low selectivity of spectrophotometry, which is usually considered as a disadvantage of this method, is improved by the narrow selection of the wavelength and working pH value. A special challenge was the selectivity in determination of morin against structurally very similar flavonol quarcetin, achieved due to fact that quarcetin makes a stable complex with zinc (II) at pH = 5.25 (32) Although some other methods, like spectrofluorimetry or even HPLC, are characterized by lower limits of detection and quantification, LOD and LOQ of the proposed spectrophotometric method are low enough for the determination of morin in real samples. Those characteristics give the developed method a promising perspective for its routine

application in the quantification of morin in fresh or processed fruit and vegetable products, such as fruit juices or supplements.

The fact that the contents of bioactive compounds in plants are influenced by the geographical region, climate, soil type, cultivar methods, growing season, harvest date and storage conditions (46-49), does not call into question the advisability of methods for quantification of flavonoids, such as morin.

DPPH and FRAP tests have shown that the antioxidative ability of strawberries and morin differ. For example, a solution of morin in a concentration of 5×10^{-6} M exhibits an activity of RSC=31.02%, corresponding to trolox concentration of 2.36 mg L^{-1}, much higher than for strawberry extract, implying that other components, such as vitamin C, contribute to the antioxidative potential of strawberries to a great extent.

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Spektrofotometrijsko određivanje sadržaja morina u jagodama i njihove antioksidantne aktivnosti

Leposava Pavun[1], Aleksandra Janošević-Ležaić[1], Snežana Uskoković-Marković[2,*]

[1]Univerzitet u Beogradu - Farmaceutski fakultet, Katedra za fizičku hemiju i instrumentalne metode, Vojvode Stepe 450, 11221 Beograd, Srbija

[2]Univerzitet u Beogradu – Farmaceutski fakultet, Katedra za analitičku hemiju, Vojvode Stepe 450, 11221 Beograd, Srbija

[*]Autor za korenspodenciju: Snežana Uskoković-Marković,
E-mail: snezaum@pharmacy.bg.ac.rs

Kratak sadržaj

Morin je jedan od flavonoida sa vrlo izraženom antioksidantnom aktivnošću. Sa ciljem korišćenja njegovih pozitivnih efekata na ljudsko zdravlje, u porastu je trend interesovanja za njegov sadržaj u hrani ili suplementima. U radu je predstavljena jednostavna i pristupačna spektrofotometrijska metoda određivanja morina, zasnovana na formiranju kompleksa morina sa Zn^{2+} jonom (u stehiometrijskom odnosu 1 : 1, na pH 7,98 i 392 nm), što joj daje prednost u odnosu na druge metode koje se mogu koristiti za kvantifikaciju morina. Metoda pokazuje linearnost odgovora u koncentracionom opsegu 0,151 – 4,533 mg L^{-1}, pri čemu limit detekcije (LOD) iznosi 0,030 mg L^{-1} a limit kvantifikacije metode (LOQ) je 0,091 mg L^{-1}. Razvijena metoda je uspešno primenjena za određivanje sadržaja morina u jagodama. Takođe, prikazani su i komentarisani rezultati određivanja antioksidativne aktivnosti jagoda i morina, DPPH i FRAP testovima.

Ključne reči: morin; jagode; spektrofotometrija; cink kompleks.