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ZINC COMPLEX-BASED DETERMINATION OF RUTIN IN DIETARY SUPPLEMENTS

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The aim of this study was to develop and validate a simple, rapid, sensitive and low-cost method for determination of rutin in tablets. The proposed spectrophotometric method is based on the formation of the Zn^{2+}-rutin complex in methanol 70% (v/v) at pH 8.52, and detection at λ_{max}= 410 nm. The concentration range over which the response was linear was 0.3–12.2 μg ml^{-1}. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.21 μg ml^{-1} and 0.63 μg ml^{-1}, respectively. The proposed method was successfully applied to the determination of rutin in herbal dietary supplements. The reliability of the method was checked by comparison with results obtained by the established RP-HPLC/UV method. The proposed method fulfills all aimed requirements.

Key words: flavonoids; rutin; zinc complex; spectrophotometric determination

ОПРЕДЕЛУВАЊЕ НА КОМПЛЕКС НА ЦИНК ВО ДИЕТЕТСКИ ДОДАТОЦИ

Целта на ова истражување беше да се разработи и валидира едноставен, брз, сензитивен и евтин метод за определување на рутин во таблети. Предложениот спектрофотометрски метод се базира на образување комплекс на Zn^{2+} во метанол 70% (v/v) на pH 8,52 и на бранова должина на определување од λ_{max}= 410 nm. Концентрацискиот опсег каде што одговорот е линеарен е 0,3–12,2 μg ml^{-1}. Прагот на детекција (LOD) и границата на квантификација (LOQ) се 0,21 μg ml^{-1} и 0,63 μg ml^{-1}, соодветно. Предложениот метод успешно е применет за определување на рутин во хербални диететски додатоци. Исправноста на методот беше проверена со резултати добиени со постојниот RP-HPLC/UV метод. Предложениот метод ги задоволува поставените барања.

Клучни зборови: флавоноиди; рутин; комплекс на цинк; спектрофотометриско определување

1. INTRODUCTION

Flavonoids are secondary metabolites of plant cells and have well-known roles in plant growth, photosynthesis, reproduction, phosphorylation of proteins and pigmentation. Flavonoids also contribute to plant resilience by fighting the negative effects of pathogens and UV radiation [1, 2].

The positive effects of flavonoids on human health are numerous, as they exhibit anti-allergic, anti-inflammatory, antioxidant, antibacterial, antifungal, antiviral, and anti-cancer properties, among others [3]; thus flavonoids are essential ingredients of nutraceuticals. Most of the advantageous health effects of flavonoids are ascribed to their antioxidant and chelating abilities [4, 5]. Metal ion complexes of flavonoids have been reported and in some cases, the formation of such complexes changes the anti-oxidative ability of the flavonoid itself [6, 7].

Rutin is a 3-glycoside derivative of the flavonol quercetin and the disaccharide rutinose (α-L-rhamnopyranosyl-(1→6))-β-D-glucopyranose) (Fig. 1). The Zn-rutin complex is well defined and characterized by several methods [8, 9]. The content of

the complex depends on solution conditions such as pH, the parent chemicals, solvents, etc. The influence of metal ion complex formation on the antioxidant effects of rutin has been examined with the aim to investigate its prospective use in therapeutic applications [8, 10].

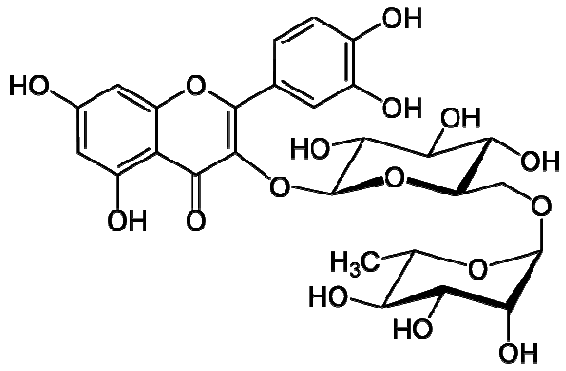

Fig. 1. Structure of rutin

Flavonoid-metal complexes possess superoxide dismutase-like behaviour, helping in superoxide anion elimination processes. It has been confirmed that metal complexes of rutin are able to neutralize superoxide anion effects [11].

The number of available nutraceuticals has rapidly increased in previous decades. Still, there is a need to develop simple low-cost methods for quality control of dietary supplements and other herbal based products containing flavonoids. This work presents a Zn-flavonoid complex based spectrophotometric determination of rutin in oral dosage formulations.

2. EXPERIMENTAL SECTION

2.1. *Materials and solutions*

Analytical grade zinc-chloride (Fluka AG, Buchs, Germany), rutin-dihydrate ($C_{15}H_{10}O_7 \cdot 2H_2O$, MW = 338.27 g mol^{-1}, CAS number 6151-25-3, Fluka AG, Buchs, Germany), acetonitrile (J.T. Baker, Deventer, Netherlands), methanol, NaOH, and CH_3COOH (Merck, Darmstadt, Germany) were used without further purification. The stock solution of zinc-chloride was prepared by dissolving $ZnCl_2$ in double-distilled water and the solution was standardized gravimetrically. The solution of rutin-dihydrate was prepared by dissolving an accurately weighed quantity of rutin-dihydrate in methanol 70% (v/v). These solutions were stored in a refrigerator. All working solutions were prepared by dilution of 1×10^{-4} mol dm^{-3} $ZnCl_2$ and 1×10^{-4} mol dm^{-3} rutin-dihydrate, respectively. The pH of all solutions was adjusted using HCl or NaOH solutions.

2.2. *Apparatus*

Spectrophotometric measurements were performed on a Beckman DU-650 spectrophotometer, using 1 cm quartz cells. The pH values were measured using a pH-meter (pHM-28 Radiometer) and combination electrode (accuracy of ±0.01 pH unit). Chromatographic measurements were carried out using an HPLC system (Shimadzu, Kyoto, Japan) composed of a quaternary pump (LC-20AT) and autosampler (injection volume 20 µl), and equipped with a Luna C_{18} column (250 × 4.6 mm, 5 µm, Phenomenex, California, USA), degasser (DGU-20A_3), column thermostat (CTO-20A) and variable UV-vis diode array (SPD-M20A). Acquisition and data analysis were performed with manufacturer software, LC Solution. LC conditions were as follows: water (mobile phase A) and acetonitrile (mobile phase B) were mixed in a linear gradient (0–5 min 90:10 A:B; 25–27 min 10:90 A:B; 28–30 min 90:10 A:B) with a flow-rate of 0.7 ml min^{-1}. The injection volume was 20 µl and the wavelength of detection of 257 nm.

2.3. *Analysis of rutin in pharmaceutical dosage forms*

2.3.1. *Pharmaceutical tablets*

Pharmaceutical tablets containing rutin were analyzed by the proposed methods and were as follows: Acerola plus® tablets (Natural Wealth, Bohemia, NY 11716, USA, nominal composition declared per tablet: vitamin C 100 mg, rutin 2.5 mg, hesperidin 1.8 mg, excipients: saccharose, microcrystalline cellulose, methylhydroxypropyl cellulose, magnesium stearate, starch, glycerin); vitamin C 1500 tablets (American Nutrition Products, nominal composition declared per tablet: vitamin C 1500 mg, rutin and hesperidin not declared); and Helopyrin tablets (Rösch & Handel, Wien, Austria, nominal composition declared per tablet: vitamin C 120 mg, bioflavonoids 20 mg, rutin 15 mg, excipients: microcrystalline cellulose, methylhydroxypropyl cellulose, magnesium stearate, starch hydrolysate).

2.3.2. *Sample preparation and analysis*

Ten tablets from each source were weighed and powdered using mortar and pestle. A portion of the powder, equivalent to the weight of one tablet, was dissolved in 100 ml methanol 70%

(v/v), and sonicated for 15 min in an ultrasonic bath at 25^{0}C. The solution was filtered through a millipore membrane filter with pore size 0.45 μm. Appropriate volumes of each filtrate and a 3.0 ml portion of 1×10^{-4} mol l^{-1} $ZnCl_2$ were prepared in volumetric flask of 10 ml and diluted to the mark with methanol 70% (v/v) so that the concentration of rutin in prepared solutions was approximately 5×10^{-6} M (e.g., 0.00305 g l^{-1}). Values of pH of these solutions were adjusted to 8.52 using HCl or NaOH. Absorbance spectra of prepared solutions were measured at λ_{max}= 410 nm. The blank was methanol 70% (v/v) pH 8.52.

3. RESULTS AND DISCUSSION

3.1. *Zinc complex of rutin*

3.1.1. *Absorption spectra*

Rutin and Zn^{2+} formed a complex in methanol 70% (v/v) in the range of pH 7.50–9.00. The absorption spectra were recorded using solutions of 2.5×10^{-6} mol dm^{-3} $ZnCl_2$, 5.0×10^{-5} mol dm^{-3} rutin, and the mixture of rutin and Zn^{2+} where the concentrations of components are the same as in the single solutions at the constant pH values (8.52). Rutin and hesperidine are usually both present in pharmaceutical preparation and dietary supplements, which may pose a problem in rutin quantification. Because of their similar chemical structure and ability to form complexes with metal ions under similar conditions, it was necessary to define the conditions where rutin selectively formed a complex with Zn^{2+}. Under those specific conditions (pH = 8.52), the very broad and intense absorption spectra of rutin (λ_{max} = 360 nm) and hesperidine (λ_{max} = 283 nm) are overlapped. The calculated spectrum of the complex (λ_{max} = 410 nm) is obtained by subtracting the corresponding absorbance of solutions of Zn^{2+} and rutin from the absorbance of their mixture, at different wavelengths. According to our preliminary tests, the complex of the Zn^{2+} with hesperidin is formed at much lower pH with λ_{max} about 300 nm, allowing spectrophotometric determination of the content of rutin based on its zinc(II) complex.

3.1.2. *Method development*

Critical parameters for method development included composition of the solvent and pH, as well as reaction time between Zn^{2+} and rutin. Optimal values for these parameters have been determined in order to achieve maximum of absorbance.

The composition of the solvent influences the absorbance values and solubility of the complex. Solvent compositions examined were 30, 50, 70 and 90 % (v/v) of methanol. The optimal composition was determined by maximum absorbance and solubility of complex to be methanol : water = 70 : 30 (v/v).

The influence of pH on the absorbance of the zinc(II)-rutin complex was examined in the range 6.2–9.0, as is shown in Figure 2. The pH dependence of absorbance exhibits a complex shape. The optimal pH value was around 8.5, which was used for all further experiments.

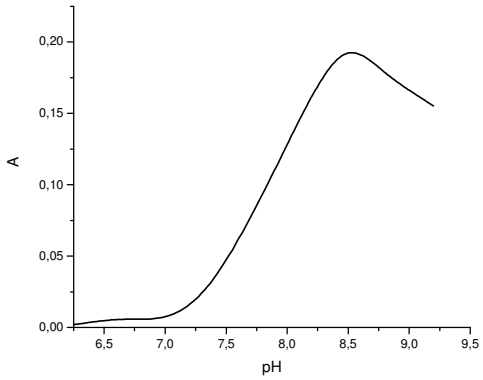

Fig. 2. Effect of pH of the absorbance of Zn-rutin complex at λ_{max} = 410 nm

3.1.3. *Composition of the complex*

The stoichiometric ratio of Zn^{2+} to rutin in the complex was determined by the method of molar ratios [12]. The absorbances of solutions containing a constant concentration of Zn^{2+} (5.0×10^{-5} mol dm^{-3}) and different concentrations of rutin ($2.5–40 \times 10^{-5}$ mol dm^{-3}) were measured at a constant pH (8.52) at 410.0 nm. A straight line, $A = f(c_{rut} / c_{Zn^{2+}})$, with intercept at $c_{rut} / c_{Zn^{2+}} = 2$ was obtained and also proved that the stoichiometric ratio of the Zn^{2+}: rutin in complex is 1 : 2.

Additionally, the composition of the complex was determined by the method of variation of equimolar solutions [13]. The absorbances of a series of solutions formed by mixing equimolar solutions of Zn^{2+} and rutin (5.0×10^{-5} mol dm^{-3}) with pH= 8.52 were measured at 410 nm. The dependence of absorbance of tested solutions on molar fractions of Zn^{2+} ion gives a curve with the maximum at x_{rut} = 0.67 (Fig. 3), corroborating the previous method, which found the complex composition was Zn^{2+}: rutin = 1 : 2. The stability constant of the complex at pH = 8.52 was estimated

from a Job plot and the (conditional) stability constant was found to be log $K = 10.86 \pm 0.02$.

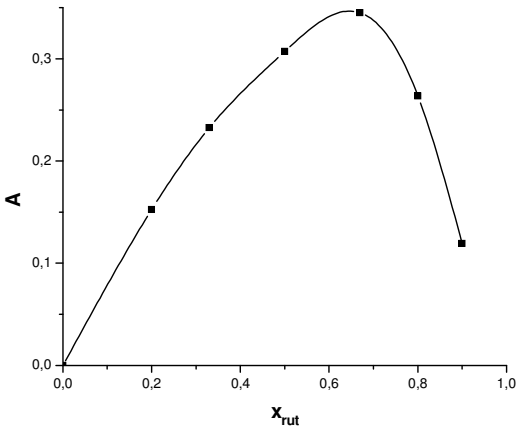

Fig. 3. Method of continual variations of equimolar solutions. Dependence of absorbance on mole fraction of x_{rut} at pH 8.52

The formation of a stable zinc(II)-rutin complex in a methanol solution can be utilized for quantitative determination of rutin in various matrices in pharmaceutical dosage forms.

3.2. *Spectrophotometric determination of rutin in aqueous-methanolic phase*

3.2.1. *Linearity*

The high value of the stability constant of the zinc(II)-rutin complex ensures the quantitative determination of rutin using the complex. The calibration curve method was used, requiring solutions containing constant concentration of $ZnCl_2$ and different concentrations of rutin in methanol 70% (v/v) as with pH 8.52. The blank was methanol 70% (v/v) with pH 8.52. Linear dependence of the absorbance of the complex on the concentration of rutin was obtained in the interval of 0.3–12.2 μg ml^{-1}. The regression equation: $A = 0.0147$ [rut] + 0.004 ($N = 8$) was calculated, where A is absorbance ($\lambda_{max} = 410$ nm) and [rut] is concentration of rutin in μg ml^{-1}. The good linearity of the calibration curve and negligible scatter of experimental points are represented by the high coefficient of correlation, $r^2 = 0.9986$.

Other spectrophotometric methods have been developed for the determination of rutin [14,15]. As a comparison, at pH 3.80 the linearity of spectrophotometric determination of rutin by complex formation with uranyl ions was 6.1–122 μg ml^{-1} [14]. The linearity range obtained for the present proposed method is 20 times lower, enabling quantification of much lower concentrations of rutin. Spectrophotometric determination of rutin in another reported method [15] was obtained in range 0.9–10.7 μg ml^{-1}. The method proposed herein has a broader range of linearity and allows detection of smaller quantities of rutin.

3.2.2. *LOD (limit of detection) and LOQ (limit of quantification)*

The limit of detection (LOD) [16] was calculated by establishing the minimum level at which rutin can be detected, according to formula:

$$\mathrm{LOD} = 3.3\, s_b/a$$

where s_b is the standard deviation of the intercept and a is the slope of the calibration line. It was found that LOD is 0.21 μg ml^{-1}.

The limit of quantification (LOQ) [16] was determined by using the formula:

$$\mathrm{LOQ} = 10\, s_b/a.$$

Rutin can be quantified at a concentration of 0.63 μg ml^{-1}.

3.2.3. *Precision*

The precision of the method was determined for three different rutin concentrations (Table 1) and is fairly high as indicated by low values of the standard deviation of the measurements (SD). The results obtained by the proposed procedure indicate that the method is precise for the determination of rutin in pharmaceutical dosage forms.

T a b l e 1

Precision of the assay for three different concentrations of rutin. All values derived from N = 5 independent measurements (CV is coefficient of variation)

Theoretical (μg ml^{-1})	Measured (μg ml^{-1})	Recovery (%)	SD	CV (%)
4.05	3.98	98.3	2.48×10^{-2}	0.61
8.10	8.08	99.75	2.92×10^{-2}	0.36
12.15	12.17	100.08	2.70×10^{-2}	0.22

3.3. *Spectrophotometric determination of rutin in tablets*

The established method was applied for the determination of rutin in dietary supplements Acerola plus® tablets (Natural Wealth, Bohemia USA), Vitamin C 1500 tablets (American Nutrition Products) and Helopyrin tablets (Rösch & Handel, Wien, Austria). Samples were prepared according to the procedure described in the Experimental Section and the results of the spectrophotometric determination of rutin in tablets are presented in Table 2.

T a b l e 2

The spectrophotometric determination of rutin in tablets, N=5

Pharmaceutical preparation	mg rutin / tablet		Recovery (%)	SD	CV (%)
	Declared	Measured			
Acerola plus® tablets (Natural Wealth, Bohemia USA)	2.50	2.69	105.4	2.0×10^{-2}	0.74
Vitamin C 1500 tablets (American Nutrition Products)	not declared	5.8	/	1.9×10^{-2}	0.33
Helopyrin tablets (Rösch & Handel, Wien, Austria)	15.0	15.3	102.0	3.4×10^{-2}	0.23

One of tested tablets (Helopyrin) with declared content of rutin fulfills requirements according to official regulations issued by European Medicines Agency (EMA) [17], while the content of rutin in Acerola plus® tablets exceeded the required limit (95–105 %).

To confirm the applicability of the spectrophotometric method for determination of rutin in some pharmaceutical formulations, the possible effect of excipients and other active compounds on the absorbance of the zinc(II)-rutin complex was investigated. For this aim, the mixtures of ingredients but without rutin were prepared. Each mixture, containing vitamin C, hesperidin and other declared excipients (listed in Experimental Section for each of formulations), was treated by the same procedure used for the determination of rutin in tablets, including spectrophotometric analysis. Under those conditions, the tested mixture showed negligible absorbance. Under our experimental conditions (pH 8.52 in methanol 70 % (v/v)), the complex of Zn^{2+} with hesperidin does not produce measurable absorbance, and thus, hesperidin does not interfere with rutin determination.

3.4. *RP-HPLC determination of rutin in tablets*

To compare the performance of the proposed spectrophotometric method for the determination of rutin in dietary supplements, a comparative method of RP-HPLC with UV-VIS detection was performed. Figure 4 presents the chromatogram of rutin recorded under the defined optimal conditions. The peak of rutin appears on the chromatogram with retention time 13.42 min.

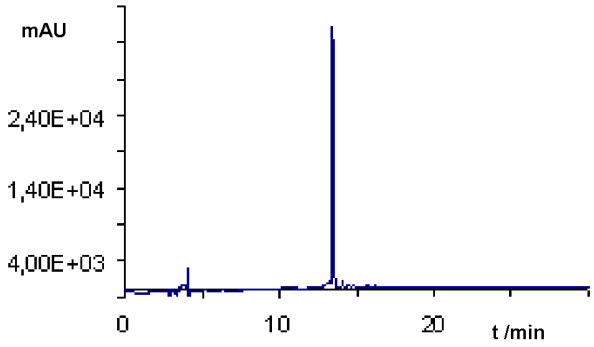

Fig. 4. Chromatogram of rutin

Based on the obtained calibration curve for rutin determination by the RP-HPLC method, the regression equation is as follows:

$$Ar = (3.29[rut] - 0.42) \times 10^4 \quad N = 8,\ r^2 = 0.9958,$$

where Ar is peak area. Linearity is achieved for the concentration range of [rut] = 0.05–10.0 μg ml^{-1}. The LOD and LOQ were calculated from the calibration curve parameters and were 0.037 μg ml^{-1} and 0.11 μg ml^{-1}, respectively. Samples of tested formulations for RP-HPLC analyses were prepared according the same procedure as described for the spectrophotometric method. The developed RP-HPLC/UV method was applied to the determination of rutin in tablets, and the results are presented in Table 3.

Like the developed spectrophotometric method, the content of rutin determined by RP-HPLC/UV method in Helopyrin tablets fulfills requirements according to EMA official regulations [17], while the content of rutin in Acerola plus® exceeded the required limit.

T a b l e 3

Results of determination of rutin by RP-HPLC/UV method, N=3

Pharmaceutical preparation	mg rutin / tablet		Recovery (%)	SD	CV (%)
	Declared	Measured			
Acerola plus® tablets (Natural Wealth, Bohemia USA)	2.50	2.65	106.0	1.8×10^{-2}	0.72
Vitamin C 1500 tablets (American Nutrition Products)	not declared	5.92	/	1.8×10^{-2}	0.30
Helopyrin tablets (Rösch & Handel, Wien, Austria)	15.0	14.46	96.4	2.3×10^{-2}	0.15

Comparing the precision of two methods, the results of an *F*-test (F_{calc}=1.235 for Acerola plus® tablets, F_{calc}=1.114 for Vitamin C 1500 tablets and F_{calc}=2.185 Helopyrin tablets) are much lower than the tabulated value at 95% confidence level (F_{tab}=6.944), and confirmed that the precision of the two methods are comparable.

4. CONCLUSIONS

The proposed spectrophotometric method for rutin determination in pharmaceutical dosage forms is simple, low cost, accurate, and precise, with high reproducibility, and it enables the direct and simple determination of rutin without its prior extraction from samples. The developed spectrophotometric determination has satisfied LOD and LOQ values comparable to those obtained by the RP-HPLC/UV method. There was no interference from excipients in the examined products, thus no additional extraction or separation procedures were required. In comparison to spectrophotometric methods reported in the literature, this method is very fast and simple to perform, with high sensitivity, wide linear range and good operational stability. Using the proposed technique, is possible to determine rutin content in pharmaceutical dosage forms if there is no presence of compounds which could react with Zn^{2+} and/or cause considerable absorbance at λ_{max} = 410 nm.

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