

## Research Article

Kristína Krajčíková\*, Mária Suváková, Gabriela Glinská, Jana Ohlasová, Vladimíra Tomečková

# Stability of natural polyphenol fisetin in eye drops

## Stability of fisetin in eye drops

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**Abstract:** Fisetin is a polyphenolic compound with anti-inflammatory and antioxidant properties. Inflammation and reactive oxygen species play a major role in the pathophysiology of the dry eye syndrome (DES). Patients with DES undergo symptomatic treatment using eye drops known as artificial tears. Addition of fisetin into the eye drops could result in a better recovery of the eye surface. This experimental study examines the stability of fisetin in selected eye drops (Arufil, Hypromelóza-P, Ocutein, Refresh). Absorption spectra of fisetin were measured in selected eye drops, dimethylsulphoxide (DMSO), deionized water and normal saline solution (NSS) during a period of four weeks. The fisetin absorption maximum was placed at 350 – 390 nm depending on the solvent. Good stability of fisetin solutions were observed in DMSO and deionized water. The highest stability of fisetin in selected eye drops was observed in Hypromelóza-P. Irreversible fisetin structural changes were detected in Arufil, Ocutein, Refresh and NSS. For further clinical evaluation, fisetin solution in Hypromelóza-P could be examined.

**Keywords:** fisetin; dry eye syndrome; stability; pH.

## 1 Introduction

Flavonols have attracted attention as new therapeutic agents thanks to their antioxidant and anti-inflammatory characteristics. They show a broad spectrum of biological activities emerging from their structure. They possess the ability to donate hydrogen of hydroxyl groups or an electron from aromatic system and, in this manner, interact with the reactive oxygen species (ROS) in their close vicinity. Delocalization of a radical electron prevents formation of another free radical [1]. Furthermore, they work as a chelation agent. Its binding of  $\text{Fe}^{2+}$  leads to the reduction of Fenton reaction products (reactive hydroxyl radicals) [2]. Moreover, they serve as enzyme and transcription factor activators that play an important role in prevention and treatment of numerous diseases [3–5]. Their stability is influenced by environmental factors. Light [6], high temperature and alkaline pH enhance degradation [7]. Hydroxylation of flavonols plays a major role in their stability. Glycosylation of these hydroxyl groups also increased stability of flavonols [7]. Moreover, the presence of aromatic system causes specific coloration and enables detection of structural changes using UV-Vis spectral methods. Flavonols provide characteristic absorption bands with the maximum of approximately 380 nm.

Fisetin is a natural 3, 7, 3', 4' – tetrahydroxyflavone (Figure 1). It possesses wide range of bioactive properties: anti-inflammatory [8], antioxidant [9], antiviral [10], antiaging [11], anticancer [12] and neuroprotective [13]. It also affects glucose metabolism [14] and angiogenesis [15]. Hytti et al. (2017) [16] proved that fisetin has anti-inflammatory effects on retinal pigment epithelium and it also prevents ischemia-induced cell death of retinal ganglion cells [17]. Furthermore, it inhibits UV-radiation induced oxidative stress in lens epithelium [18].

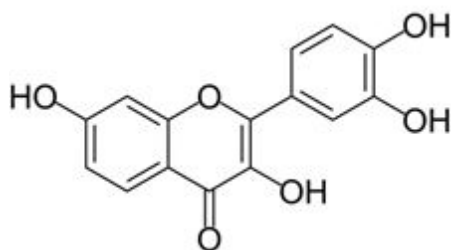
Currently, eye drops enriched by various antioxidants (vitamin A, C, E and others) are available. Therefore, flavonoids have started to gain attention in the treatment of ocular surface. They help to nourish avascular cornea and are used for prevention or treatment of the dry eye syndrome (DES). DES is highly prevalent worldwide and

**\*Corresponding author: Kristína Krajčíková**, Department of Medical and Clinical Biochemistry, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Košice, 04011, Slovakia, E-mail: kristina.ugrayova@student.upjs.sk

**Mária Suváková**, Department of Biochemistry, Institute of Chemistry, Faculty of Science, Pavol Jozef Šafárik University in Košice, Košice, 04154, Slovakia

**Gabriela Glinská**, Ophthalmology Clinic, PROOKO, spol. s r.o., Košice, 04001, Slovakia

**Jana Ohlasová, Vladimíra Tomečková**, Department of Medical and Clinical Biochemistry, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Košice, 04011, Slovakia



**Figure 1:** Fisetin structure. Fisetin consists of two phenyl rings and one heterocyclic ring with hydroxyl groups on C3, C7, C3', C4'.

reduces life quality [19]. It has impact on daily tasks such as reading and driving and the treatment available can only ameliorate the symptoms. The more potent substance without side effects is needed to fight inflammatory processes and the formation of reactive oxygen species which takes part in pathogenesis of DES. In the eye, the fisetin anti-inflammatory effect persists in various model systems [16].

The new active pharmaceutical ingredient undergoes a series of tests. Stability is the fundamental parameter in the establishment of new agents into the pharmaceutical practice. In the field of ophthalmology, active ingredients' stability in eye drops varies. It could be limited to several days in the case of antibiotic agents, but 30-days persistence has been observed in other types of eye drops. Stability tests include physical, chemical and microbiological assessment. Physical assessment includes olfactory and visual analysis, chemical evaluation comprises monitoring of active compound concentration, degradation products, pH and osmolality and microbiological examination where bacterial growth is excluded. After meeting these criteria, incorporation of an active ingredient and creation of eye drops is possible under the aseptic conditions required for the topical eye application products, given that an eye is a sensitive organ.

Dimethylsulphoxide (DMSO) is an aprotic clear odourless solvent which solubilizes the wide range of both polar and non-polar compounds. As a variety of pharmaceutical active agents are poorly soluble in water, the medical use of DMSO as a solvent and carrier has started to rise. It was applied therapeutically for the first time to women with interstitial cystitis and since then, it has been tested for skin, oncological and cardiovascular diseases [20]. However, despite its beneficial effect, the total amount of DMSO in a drug must be strictly determined. While the use of low DMSO content did not result in a significant negative outcome on cell viability, morphology and inflammatory status [21, 22], its high content (>10%) promoted harmful events and biological

membranes disruption [23]. The effect on the eye surface is also controversial. While Altan and Oğurtan (2017) [24] showed that 40% DMSO induced reepithelization on eye burns, Galvao et al. (2014) [25] demonstrated that even 2-4% DMSO induced cell apoptosis and represents a potential risk. In the cell assays, the save and commonly used total DMSO content is under 0.5%. Careful examinations should be conducted during establishment of DMSO as an ingredient for topical eye treatments.

This study deals with the stability of fisetin dissolved in DMSO in various eye drops and represents the possible strategy of fisetin administration to the eye.

## 2 Methods

### 2.1 General Experimental Procedures

UV-Vis spectra measurements were conducted on SPECORD S300 UV VIS (Analytik Jena). Hanna Instruments HI 2211 pH/ORP Meter was used to measure pH.

Fisetin was purchased from Santa Cruz Biotechnology, Inc. (Germany). Dimethylsulphoxide was obtained from Merck KGaA (Germany), normal saline solution from Bieffe Medital (Italy).

Arufil (povidonum, benzalkonii chloridum, dinatrii edetas dihydricus, dinatrii hydrogenphosphate dodecahydrate, natrii dihydrogenphosphate dihydrate, natrii chloridum, acqua ad injectabilia) was acquired from Bausch&Lomb (USA), Hypromelóza-P (hypromellose, dexpanthenolum, benzalkonii chloridum, natrii chloridum, dinatrii edetas dihydricus, aqua ad injectabilia) from Unimed Pharma (Slovakia), Ocutein (acqua, acidum boricum, natrii chloridum, polyhexamethylene biguanid, borax, dinatrii dihydro-ethylendiamintetraacetat, natrii hyaluronas) from Simply You Pharmaceuticals (Czech republic), Refresh (natrii carboxymethylcellulosum, PURITE®) from Allegran (Ireland). All eye drops were stored according to manufacturer instruction.

Resistivity of deionized water was 10 MΩ.cm.

### 2.2 Preparation of fisetin solutions

The 10 mM fisetin stock solution was prepared in DMSO. Subsequently, the respective volume of eye drops and fisetin stock solution were mixed to obtain final fisetin concentrations of 25 μM and 50 μM with the total DMSO content of 0.5%. The solutions were stored in dark at ambient temperature for a period of four weeks.

## 2.3 UV-Vis spectrophotometry

UV-Vis spectra measurements were conducted with the following settings: Wavelength range was 200-600 nm, integration time 442 ms and the number of accumulations 10. Dark current correction was used and spectrum mode was selected. Spectra were measured in quartz cuvette with optical path length of 1 cm at 25°C. Each sample was measured on the day of preparation and then after 7, 14, 21 and 28 days and spectral changes were monitored.

## 2.4 Reversibility test of fisetin changes in eye drops

For testing whether the observed spectral shifts correspond to reversible or irreversible fisetin structural changes in eye drops, the spectra of fisetin in Arufil, Ocutein and Refresh were measured after 1:1 dilution of 50  $\mu$ M fisetin sample with DMSO or deionized water at 25°C and in the presence of oxygen. After mixing up, a sample of 25  $\mu$ M fisetin and 50% DMSO or water content were obtained and subsequent spectral changes were observed.

## 2.5 pH measurements and buffer solutions

Glass body refillable pH semi-micro electrode with Ag/AgCl reference was used (Hanna Instruments HI 1131 B). For pH-dependent spectral changes observations 200 mM phosphate buffers (Sigma-Aldrich, Germany) with pH 6.12 and 7.99 (23°C) were used.

## 2.6 Data analysis

The data analysis was performed using the OriginPro 8.5 software (OriginLab Corp.).

Ethical approval: The conducted research is not related to either human or animal use.

# 3 Results

During four weeks, a negligible shift appeared in the wavelength of fisetin absorbance maximum in DMSO (after 0 days: 363 nm, after 7, 14, 21 and 28 days: 362 nm), Hypromelóza-P (after 0 days: 362 nm, after 7, 14, 21 and 28 days: 360 nm) and deionized water (after 0 days: 359 nm, after 7 days: 358 nm, after 14, 21 and 28 days: 357 nm) for both fisetin concentrations and in NSS (after 0 days: 358

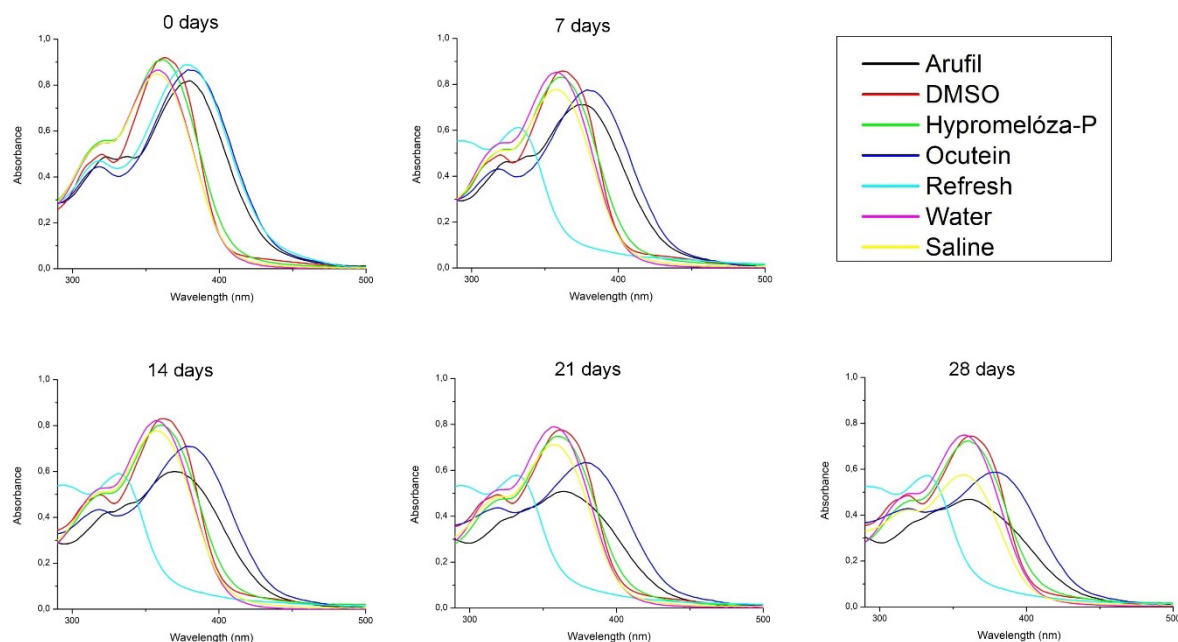
**Table 1:** Absorbance of 50  $\mu$ M fisetin main peak in the eye drops (Arufil, Refresh, Ocutein, Hypromelóza-P), dimethylsulphoxide (DMSO), deionized water and normal saline solution (NSS) after 0, 7, 14, 21 and 28 days.

| Solvent                | 0 days | 7 days | 14 days | 21 days | 28 days |
|------------------------|--------|--------|---------|---------|---------|
| Arufil                 | 0.818  | 0.710  | 0.599   | 0.507   | 0.468   |
| DMSO                   | 0.919  | 0.857  | 0.829   | 0.776   | 0.743   |
| Hypromelóza-P          | 0.911  | 0.830  | 0.801   | 0.747   | 0.723   |
| Ocutein                | 0.866  | 0.775  | 0.709   | 0.633   | 0.586   |
| Refresh                | 0.888  | 0.611  | 0.590   | 0.578   | 0.572   |
| Deionized water        | 0.865  | 0.852  | 0.821   | 0.789   | 0.748   |
| Normal saline solution | 0.847  | 0.777  | 0.777   | 0.711   | 0.574   |

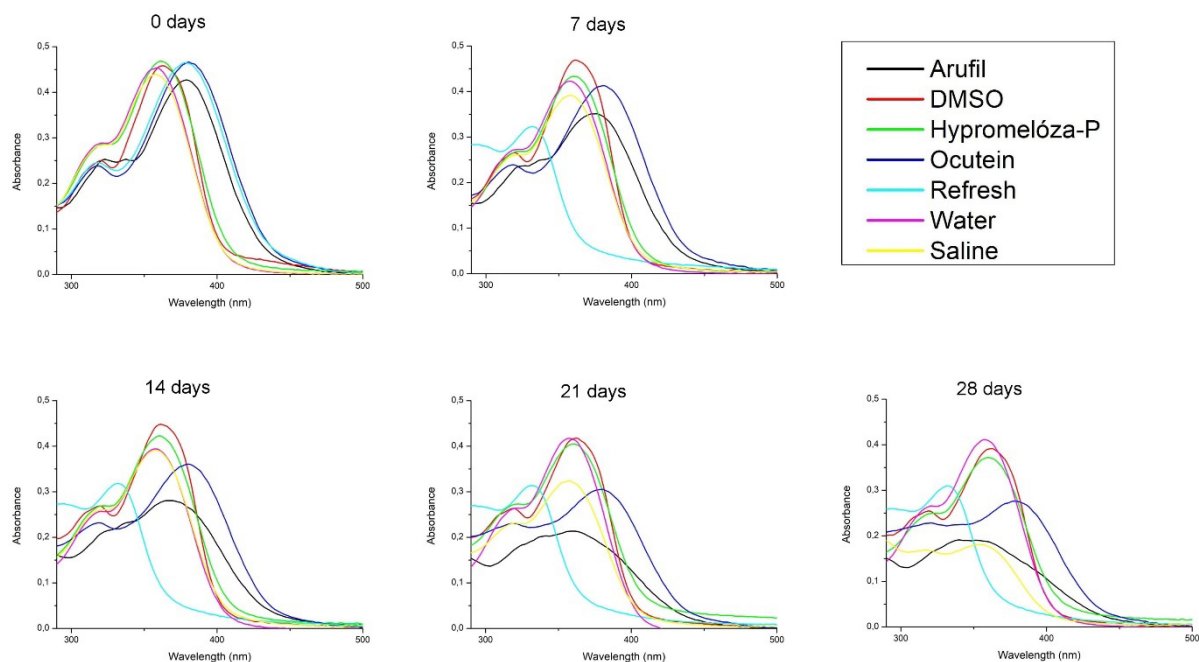
nm, after 7, 14, 21 and 28 days: 357 nm) for 50 mM fisetin concentration (Figure 2-3). In these solutions, fisetin shows good stability manifested also by small decrease in absorbance maximum (Table 1-2).

In Arufil, Ocutein, Refresh for both fisetin concentrations and NSS for 25 mM fisetin concentration, changes in fisetin spectra shape and shifts of the absorbance maximum wavelength occurred (Figure 2-3). Fisetin absorbance spectra in Arufil, Ocutein and Refresh were red-shifted for both fisetin concentrations at the beginning of the experiment (after 0 days). In the following four weeks, fisetin spectrum in Arufil changed for both fisetin concentrations. The main peak at 379 nm shifted to 378 nm after the first week, to 366 nm after the second week, to 360 nm after the third week and to 340 nm after the fourth week for 25 mM fisetin concentration. For the 50 mM fisetin concentration, the main peak at 380 nm shifted to 373 nm after the first week, to 370 nm after the second week, to 364 nm after the third week and to 362 nm after the fourth week. A new shoulder also appeared for both fisetin concentrations. Fisetin spectrum in Ocutein eye drops did not show any shifts in the absorbance maximum or the shape during four weeks for both fisetin concentrations. Fisetin spectrum in Refresh showed significant blueshift after the first week for both fisetin concentrations. The spectrum of 25 mM fisetin in NSS changed markedly after four weeks. This change was not observed for the 50 mM fisetin concentration. Furthermore, considerable decrease in the absorbance of fisetin main peak occurred in the same solvents for both fisetin concentrations (Table 1-2).

This is in accordance with the examination of reversibility of fisetin spectral changes (Figure 4). The addition of DMSO to the freshly prepared fisetin solution in

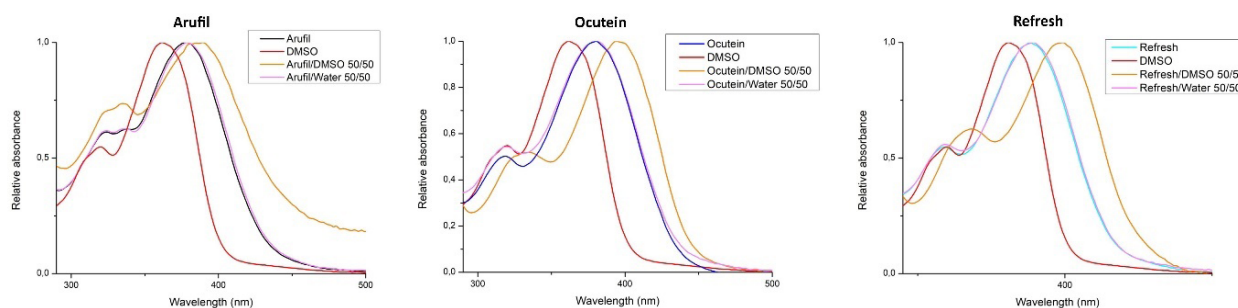


**Figure 2:** Absorption spectra of 50 M fisetin in eye drops (Arufil, Hypromelóza-P, Ocutein, Refresh), dimethylsulphoxide (DMSO), deionized water and normal saline solution (NSS) during four weeks. Fisetin was dissolved in DMSO (stock solution) and the solution was mixed with the respective volume of eye drops, DMSO, deionized water or NSS to obtain final 50 M fisetin concentration. Absorption spectra of fisetin in eye drops, DMSO, deionized water and NSS have been measured on the day of solutions preparation (0 days), after 7, 14, 21 and 28 days.



**Figure 3:** Absorption spectra of 25 M fisetin in eye drops (Arufil, Hypromelóza-P, Ocutein, Refresh), dimethylsulphoxide (DMSO), deionized water and normal saline solution (NSS) during four weeks. Fisetin was dissolved in DMSO (stock solution) and the solution was mixed with the respective volume of eye drops, DMSO, deionized water or NSS to obtain final 25 M fisetin concentration. Absorption spectra of fisetin in eye drops, DMSO, deionized water and NSS have been measured on the day of solutions preparation (0 days), after 7, 14, 21 and 28 days.





**Figure 4:** Reversibility of fisetin spectral changes. Fisetin spectra in dimethylsulphoxide (DMSO), Arufil, Ocutein and Refresh before and after addition of DMSO or water in 50/50 ratio to particular eye drops. The spectra of fisetin in Arufil, Ocutein and Refresh were measured after 1:1 dilution of 50  $\mu\text{M}$  fisetin sample with DMSO or deionized water. After mixing up, the spectra of final 25  $\mu\text{M}$  fisetin and 50% DMSO or deionized water content were obtained. Spectral shifts of fisetin absorption peak in Arufil, Ocutein or Refresh to wavelength relative to DMSO or deionized water were not observed suggesting irreversibility of the detected changes.

Arufil, Ocutein and Refresh shifted the spectrum towards higher wavelengths in all three eye drops types. Addition of deionized water did not result in spectrum shift.

For examination of the pH effect on the fisetin structure, pH values of the solvents were measured (Table 3) together with absorption spectra of 50 mM fisetin solution in phosphate buffers at pH 6.12 and 7.99 (Figure 5). The main peak of fisetin in phosphate buffer pH 6.12 was at 357 nm and for pH 7.99 was at 381 nm.

## 4 Discussion

The data concerning the therapeutic effect of natural compounds with weak solubility in aqueous solutions are inconsistent [26-28]. One of the reasons for such inconsistency is different preparation procedures with subsequent different bioavailability. In the study, we focused on fisetin which belongs to compounds weakly soluble in aqueous solutions (in water 10.45 mg/ml, in ethanol ~5 mg/ml and in dimethylsulphoxide (DMSO) (~30 mg/ml)) [29]. To dissolve fisetin in eye drops, DMSO was chosen for preparation of fisetin stock solution. Although high DMSO concentration is harmful for living organisms, a positive cellular response was observed up to 0.5 % [30]. In our formulation, the total DMSO content did not exceed this level.

The fisetin spectra in selected eye drops differed. The rationale for redshifts in absorption spectra are changed electron transitions. Fisetin consists of aromatic rings rich on  $\pi$  electrons, undergoes keto-enol tautomerism and forms radicals during elimination of ROS [31]. All such electron rearrangements are manifested as changes in the absorption spectrum [32, 33]. The fisetin electron changes

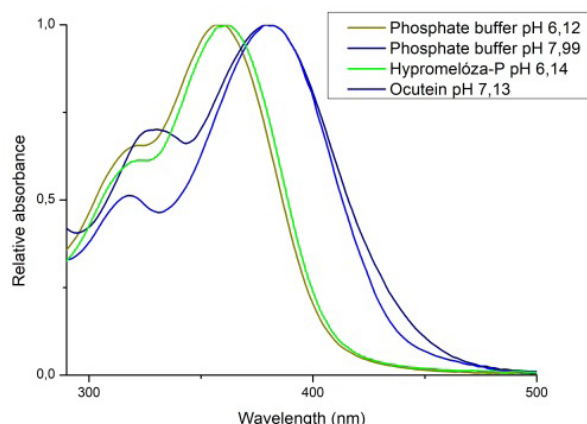
**Table 2:** Absorbance of 25  $\mu\text{M}$  fisetin main peak in the eye drops (Arufil, Refresh, Ocutein, Hypromelóza-P), dimethylsulphoxide (DMSO), deionized water and normal saline solution (NSS).

| Solvent                | 0 days | 7 days | 14 days | 21 days | 28 days |
|------------------------|--------|--------|---------|---------|---------|
| Arufil                 | 0.426  | 0.351  | 0.280   | 0.213   | 0.190   |
| DMSO                   | 0.458  | 0.469  | 0.447   | 0.417   | 0.391   |
| Hypromelóza-P          | 0.467  | 0.433  | 0.404   | 0.404   | 0.371   |
| Ocutein                | 0.466  | 0.412  | 0.360   | 0.305   | 0.276   |
| Refresh                | 0.464  | 0.323  | 0.321   | 0.313   | 0.309   |
| Deionized water        | 0.453  | 0.422  | 0.394   | 0.417   | 0.411   |
| Normal saline solution | 0.439  | 0.391  | 0.391   | 0.323   | 0.180   |

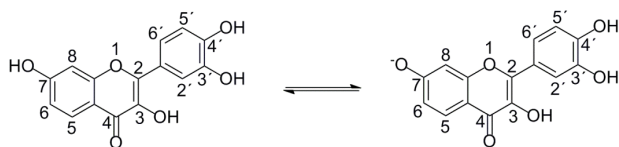
**Table 3:** pH values of eye drops (Arufil, Refresh, Ocutein, Hypromelóza-P), deionized water and normal saline solution (NSS).

| Solvent                | pH   | Temperature ( $^{\circ}\text{C}$ ) |
|------------------------|------|------------------------------------|
| Arufil                 | 7.34 | 21.6                               |
| Hypromelóza-P          | 6.14 | 21.6                               |
| Ocutein                | 7.13 | 21.5                               |
| Refresh                | 7.14 | 21.6                               |
| Deionized water        | <6   | 21.7                               |
| Normal saline solution | 5.72 | 21.8                               |

are influenced by nearby surroundings [7, 34], therefore, solvent contribution on the electron distribution in fisetin is crucial. Solvent ionic strength, pH and polarity influence the resulting spectrum [35]. However, all eye drops used



**Figure 5:** Fisetin pH-dependent spectral changes. Comparison of fisetin spectra in phosphate buffers pH 6.12, 7.99, Hypromelóza-P and Ocutein. For pH-dependent spectral changes observations, 200 mM phosphate buffers with pH 6.12 and 7.99 were used. Fisetin absorption spectra in phosphate buffer pH 6.12 and 7.99 shifted to the wavelength of Hypromelóza-P 6.14 and Ocutein 7.13, respectively.



**Figure 6:** Fisetin protonated and deprotonated species. Fisetin  $pK_a$  of OH group at C7 position is 7.27 in water environment. Various eye drops differ in pH values resulting in the different ratio between protonated and deprotonated fisetin species.

in this study were water-based, hence, their polarity did not differ significantly. Ionic strength of the eye drops is required to strictly match the tear fluid values. However, the eye can handle a wider pH range, compared to blood. Also, pH of eye drops differs, as it was shown in this study (Table 3). The most considerable fisetin spectral changes were observed in Arufil, Ocutein and Refresh eye drops with pH 7.34, 7.13 and 7.14, respectively. These values are close to fisetin  $pK_a$  of OH group at C7 position ( $pK_a = 7.27$  in water environment) [36] suggesting higher concentration of deprotonated fisetin species (Figure 6) in comparison to solvents with lower pH. Friedman and Jürgens [37] explained in detail that various polyphenolic compounds are sensitive to pH changes due to their OH groups. They described the changes as time-dependent and irreversible which is in accordance with results of this study (Figure 4). Also Jurasekova et al. [38] studied fisetin at different pH using UV Vis absorption spectroscopy, confirming fisetin oxidation products at  $pH \geq 9.2$  by Raman and

surface-enhanced Raman scattering spectroscopy (SERS). Therefore, we suppose that our conditions did not provide fisetin oxidation products, but deprotonated species.

It is not possible to completely exclude the incompatibility of excipients interactions with fisetin. For example, boric acid, the component of Ocutein, undergoes Wilson's reaction with flavonoids forming complexes [39]. However, we can keep out the substances of Arufil, Ocutein and Refresh which were common with Hypromelóza-P: preservative benzalkonium chloride and salts sodium chloride and sodium edeticum.

The decrease in fisetin absorbance maximum was presumably caused by a drop in fisetin concentration with time. The most significant declines in fisetin concentration (Arufil, Ocutein, Refresh, NSS) correlate with the spectral analysis changes.

Although fisetin is considered to have slightly weaker antioxidant capacity in comparison with quercetin [40], current studies stress the contribution brought by different experimental conditions. Fisetin showed more significant inhibition of lipid peroxidation in lung homogenates [41] and was the most potent senolytic agent in comparison with other 10 flavonoids [42]. Therefore, it is recommended to perform more tests for its utility as an active pharmaceutical ingredient in eye drops against dry eye syndrome.

To sum up, fisetin is a natural compound with high potential as a pharmacologically active substance. Its biological activity is dependent on its structure which undergoes changes in the environment of various solvents. In our study, we demonstrated changes in fisetin stability in the eye drops (Arufil, Hypromelóza-P, Ocutein, Refresh), dimethylsulphoxide (DMSO), deionized water and normal saline solution (NSS) during four weeks for 25 mM and 50 mM fisetin solutions. High stability of fisetin solutions (both concentrations) occurred in DMSO, deionized water and Hypromelóza-P. The irreversible structural changes were observed in Arufil, Ocutein, Refresh and NSS. For further ophthalmological clinical studies, fisetin solution in Hypromelóza-P is recommended.

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**Conflict of interest:** The authors declare no conflict of interest. Research was conducted without any commercial or financial relationships to eye drops producers.

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