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Fluorometric determination of hesperidin in orange juices available on Serbian market

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Abstract: The spectrofluorometric method based on fluorescence ability of aluminium – hesperidin complex for the determination of hesperidin in orange juice is proposed. The linearity range of hesperidin in methanolic-aqueous solution was 0.08 – 18.0 μg/mL with LOD and LOQ values as 0.023 μg/mL and 0.070 μg/mL, respectively, and recovery values in the range 97.8 – 99.7 %. Method was simplified by omitting any of surphactant agents usually applied in similar procedures, and successfully applied for the determination of hesperidin in orange juices commercially available on the Serbian market.

Key words: hesperidin, orange juice, spectrofluorometry.

Introduction

Flavonoids are a huge heterogenic group of naturally occurring phenolic compounds, attracting both the scientific and public audience due to their

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numerously reported bioactivity, such as anti-inflammatory, anti-oxidant, anti-allergic, hepatoprotective, anti-thrombotic, anti-viral, cardiovascular and anti-carcinogenic effects (Andersen and Markham 2006, Bubols *et al.* 2012, Kay *et al.* 2012, Procházková *et al.* 2011, Russo and Cesario 2012).

Numerous studies focused on effects of Hesperidin (Fig. 1), a flavanone-type flavonoid that consists of aglycone hesperitin and sugar rutinoside.

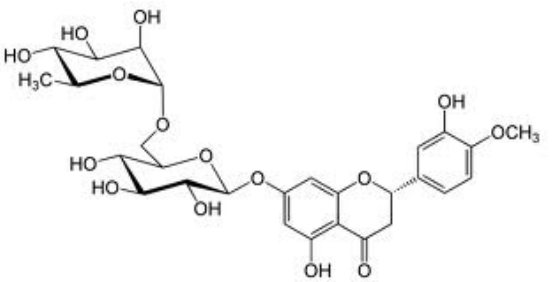

Figure 1. The molecular structure of hesperidin

Hesperidin has been reported to have antiinflammatory (Emim *et al.* 1994), antiallergenic (Lee *et al.* 2004), antihypertensive (Yamamoto *et al.* 2008), antiviral (Saha *et al.* 2009), anticoagulant (Kuntić *et al.* 2011) and vasodilatory properties (Calderone *et al.* 2004). Those effects of hesperidin are based on its strong antioxidant activity, arising through its ability to scavenge free radicals (Procházková *et al.* 2011).

A number of references report methods for the determination of hesperidin alone and/or with others flavonoids by high-performance liquid chromatography (Gorinstein *et al.* 2006, Lee *et al.* 1995, Xia *et al.* 2006), adsorptive-stripping voltammetry (Volikakis and Efstathiou 2000), spectrophotometry (Kuntic *et al.* 2012, Malešev *et al.* 1997), and pulse perturbation of the oscillatory reaction system (Pejic 2005). More sensitive methods applied for hesperidin determination in plasma include liquid chromatography tandem mass spectrometry (LC-MS/MS) (Li *et al.* 2004, Liu *et al.* 2008, Tong *et al.* 2012).

A spectrofluorimetrc method was proposed by Perez-Ruiz et al. for the determination of hesperidin based on complexation between hesperidin and aluminium(III)-ion in a micellar (sodium dodecylsulphate) medium (Perez-Ruiz et al. 1999). Our previously published work proposed a new method for the determination of hesperidin in human plasma and pharmaceutical forms based on the fluorescence properties of the aluminium-hesperidin complex in the presence of zwiterionic surfactant sulfobetaine - SB 12 (3-(N-hexadecyl-N,N-dimethylammonio) propane sulfonate) (Pavun et al. 2012). As a reference method, we used MS/MS determination of hesperidin in human plasma and a HPLC/UV method for its determination in some pharmaceutical formulations (Pavun et al. 2012). Actually,

many previously described methods reported the usage of surfactants as necessary.

In this contribution we present the application of a method based on the same principle as reported by Pavun et al. 2012, but without using the expensive SB 12 and simplifying the procedure. The method was applied for determination of hesperidin in orange juices commercially available on the Serbian market.

Reagents and Materials

Experimental work was similar to the procedures reported in our previous study (Pavun et al. 2012). Aluminium-nitrate, hesperidin (Fluka AG), methanol, NaOH, CH_3COOH (Merck) all p.a. grade, were used. The stock solution of aluminium-nitrate was prepared by dissolving $Al(NO_3)_3$ in doubly distilled water with the addition of nitric acid, while aluminium was determination gravimetrically. The stock solution of hesperidin was prepared by dissolving hesperidin in methanol (70 wt%), and was stored in a refrigerator.

A working solution (5.00×10^{-6} mol L^{-1}) of the hesperidin-aluminium complex was prepared by dilution of the stock solutions of aluminium (1.00×10^{-3} mol L^{-1} $Al(NO_3)_3$) and hesperidin (1.00×10^{-4} mol L^{-1}).

Acetate buffers (in 70 wt% methanol), previously prepared according to Perrin (Perrin 1974), were used for all spectrofluorometric measurements.

Instruments

Fluorescence spectra were collected using a Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, Paris, France) equipped with a 450 W xenon lamp and a photomultiplier tube. Samples were placed in a 1-cm optical path length quartz cuvette for spectral recording. The slits on the excitation and emission beams were both set at 5 nm. The spectra were corrected for the dark counts. The procedure involved averaging three scans with one-second integration time for each measurement. The emission spectrum of the solvent (70 wt% methanol) was subtracted. All measurements were performed at 25 ^{o}C controlled by a Peltier element. Measurements of pH were carried out using a Metler Toledo mp 120 pH meter, equipped with a combination electrode.

Sample preparation

To the aliquot of 2 mL of tested orange juice add 5.5 mL of acetate buffer pH=5.4 and centrifuge at 900 rpm for 10 min. To a 5 mL portion of the supernatant, add 0.25 mL of $Al(NO_3)_3$, c=10^{-3} M, and dilute 1 mL of the obtained mixture with 9 mL of acetate buffer; use for hesperidin measurement.

Results and discussion

Taking into account that citrus and citrus juices are naturally abounded sources of hesperidin, it is necessary to develop a simple, accurate and precise method for its determination in such samples. We found that the method we have proposed has more than satisfactory sensitivity for the routine determination of hesperidin in citrus juices, without the need for the surfactanct.

Complex formation between hesperidin and aluminium(III)-ion

Hesperidin and aluminium(III)-ion upon reaction in methanolic solution form the complex in the pH range 3.0 – 7.0. The fluorescence spectra were recorded using 70% methanol as a blank and excitation and emission wavelenghts were λ_{ex}= 390 nm and λ_{em} = 490 nm, respectively.

As described in our prior study (Pavun *et al.* 2012), the increase of intensity of fluorescence of hesperidin solution (c = 5.0×10^{-5} mol L^{-1}) upon addition of Al^{3+} ion enables adequate sensitivity of hesperidin determination in samples abounded in hesperidin such as orange juices There is the same type of response for Al^{3+} solution with the addition of varying concentrations of hesperidin, even in the absence of surfactant. The stoichiometry of the complexation was investigated by using Job method (Irving and Pierce 1959). As a conclusion, at pH 5.50 the most probable stoichiometry of the complex is Al : L = 1: 1. (Fig. 2).

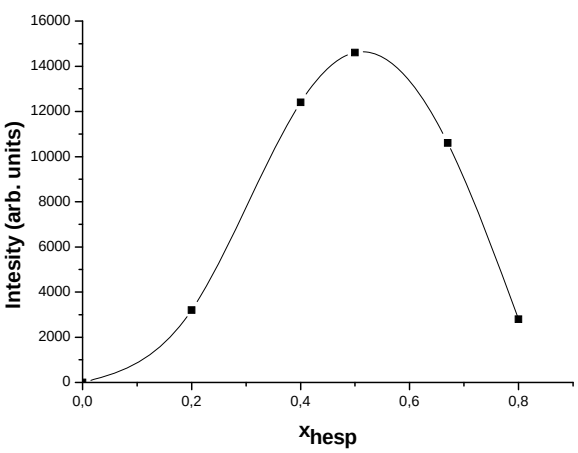

Fig. 2. Method of variations of equimolar solutions. Change of absorbance vs. mol fraction of hesperidin, x_{hesp}

The composition of the aluminium - hesperidin complex was also confirmed by the mole ratio method (Yoe and Jones 1944). The result confirms the aluminium–hesperidin ratio 1:1 for the complex formed at pH 5.50. The stability constant of the complex at pH 5.50 was estimated from the Job plot.

` The dependence of fluorescence intensity on pH was tested in the acetate buffers (in 70 wt% methanol) of different pH values, prepared according to Perrin (Perrin 1974). Fig. 3. represents the fluorescence intensity as function of solution pH.

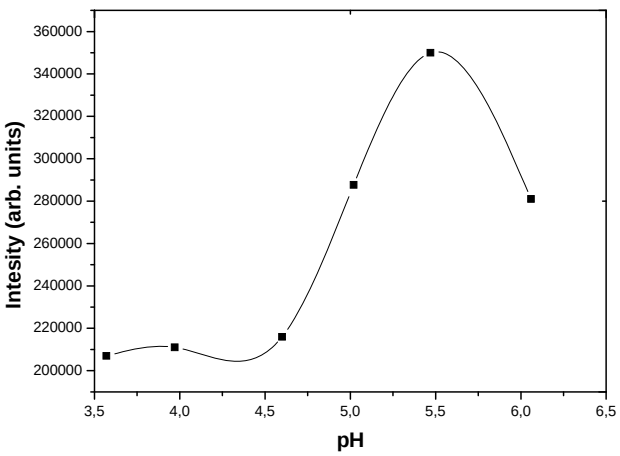

Fig. 3: Dependence intensity of fluorescence on the pH

The fluorescence intensity in this case has strong pH dependence. At low pH, it decreases because protons tend to displace Al^{3+}, while at pH > 5.50 aluminium ions form hydroxide complexes. The pH dependence differs from that with surfactant (Pavun *et al*. 2012), with the optimum pH values shifted from 4.58 to 5.50.

The quantitative spectrofluorometric determination of hesperidin

The formation of a stable aluminium-hesperidin complex in methanolic solution with enhanced fluorescence can be utilized for quantitative determination of hesperidin in orange juice in considerable amounts. Hesperidin is a main component among citrus flavonoids. For a long time, determination of hesperidin with sufficient sensitivity was reported to be possible only in the presence of surfactant.

Calibration graph in aqueous – methanolic phase

Linearity

The high value of the stability constant of the aluminium - hesperidin complex guarantees the quantitative determination of hesperidin based on the fluorescence characteristics of the complex. The calibration curve method was used, requiring solutions containing constant concentration of $Al(NO_3)_3$ and different concentrations of hesperidin in acetate buffer pH 5.50 (70 wt% methanol as solvent) and acetate buffer pH 5.50 as blank. Linear dependence of the intensity of fluorescence of the complex on the concentration of hesperidin was obtained in the interval 0.08 – 18.0 μg mL^{-1}. The regression equation:

$$I = (3.13 \pm 0.05)\, c + (0.06 \pm 0.02)$$

was calculated with the aid of Origin v. 7 software, where *I* is fluorescence intensity in % (λ_{ex} = 390 nm, λ_{em} = 490 nm) and *c* is concentration in μg mL^{-1}. The good linearity of the calibration curve and small scatter of experimental points resulted in a high coefficient of determination, $r^2 = 0.9969$.

LOD (Limit of Detection) and LOQ (Limit of Quantification)

The limit of detection (LOD) (ICH Guideline Q2B 1997, Miller and Miller 2005) was calculated by establishing the minimum level at which hesperidin can be detected, according to the formula: $LOD = 3.3\ s_b/a$, where s_b is the standard deviation in the intercept and a is the slope of the calibration line. It was found that LOD is 0.023 μg mL^{-1}.

The limit of quantification (LOQ) (ICH Guideline Q2B 1997, Miller and Miller 2005) was determined by using the formula: $LOQ = 10\ s_b/a$. Hesperidin can be quantified at a concentration of 0.070 μg mL^{-1}.

Precision

The accuracy of the method was determined for four different hesperidin concentrations (Table 1). The accuracy and repeatability of the method are fairly high, as indicated by good recovery and low values of CV.

Table 1: The spectrofluorimetric determination of hesperidin in aqueous - methanolic solutions

Taken (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)	SD	CV (%)
1.83	1.79	97.8	$2.3\ 10^{-2}$	1.31
3.05	2.99	98.0	$1.7\ 10^{-2}$	0.57
6.10	6.08	99.6	$2.2\ 10^{-2}$	0.36
12.21	12.18	99.8	$2.8\ 10^{-2}$	0.23

We may compare the parameters of spectroflurimetric determinations of hesperidin with and without surfactant SB 12 (Table 2). Obviously the surfactant SB 12 leads to better sensitivity and precision of determination, necessary for the quantification in biological fluids or pharmaceutical dosage forms. However, having in mind the expected quantity in orange juices, the procedure without very expensive SB 12 is more than satisfactory.

Table 2: Surfactant SB 12 effects on analytical parameters for spectrofluorimetric determination of hesperidin

	Method with SB 12 (*Pavun et al. 2012*)	Without SB 12
Excitation wavelength	390 nm	390 nm
Emission wavelength	476 nm	490 nm
pH	4.58	5.50
Regression equation	$I_F = (4.06\pm0.01)c + (1.27\pm0.02)$	$I_F = (3.13\pm0.05)c + (0.06\pm0.02)$
Linearity range	0.06 – 24.4 μg mL^{-1}	0.08 – 18.0 μg mL^{-1}
LOD	0.016 μg mL^{-1}	0.023 μg mL^{-1}
LOQ	0.049 μg mL^{-1}	0.070 μg mL^{-1}
Recovery	99.3 – 99.7 %	97.8-99.8 %
CV	0.13-0.81%	0.23-1.31%

Hesperidin content in orange juices available on Serbian market

The results of hesperidin spectrofluorimetric determination based on the aluminium complex for five orange juices available on the Serbian market are given in Table 3.

Tab. 3: Hesperidin content in orange juices

Juice brand	Hesperidine (g l^{-1})	SD
Life (Nectar)	0.308	0.0042
Happy day	0.350	0.0050
Next (100%)	0.317	0.0046
Pago	0.573	0.0067
Bravo	0.477	0.0042

The amounts of bioactive compounds in fruit, including citrus flavonoids, are a mulitifactorial function, depending on geographical region, climate, soil properties, type of cultivar, growing season, harvest date, storage, low-dose irradiation, and other conditions (Albach *et al.* 1981, Patil *et al.* 2004), so besides other compounds, the content of hesperidine could vary in a great range.

Further, the highest hesperidin concentrations are found to be in the peel and membranous parts of citrus. Thus, home-made juices contain very low concentrations of hesperidin. Commercial juices are rich in hesperidin because they usually include the peel constituents. The *Code of Practice for evaluation of quality and authenticity of fruit and vegetable juices,* published by the AIJN-European Fruit Juice Association, special requirements for quality of citrus based juices (oranges, lemon and grapefruit) defined the content of hesperidin as 250-700 mg/L.

Orange juices tested for hesperidin amount do not have declared content of hesperidin, so it is not possible to give the recovery values. Nevertheless, we have found, according to our results, that all tested samples have appropiate content of hesperidin in agreement with The *Code of Practice for evaluation of quality and authenticity of fruit and vegetable juices*.

Conclusion

In this work, a simple spectrofluorometric determination of hesperidin in orange juice, after its complexation with aluminium ion, provides good accuracy and precision and may be used for routine analysis. We confirmed that there is no need for any surfactant agent. All tested orange juices fulfill the requirement considered for the content of hesperidin.

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FLUOROMETRIJSKO ODREĐIVANJE HESPERIDINA U SOKOVIMA OD POMORANDŽE PRISUTNIM NA TRŽIŠTU SRBIJE

-originalni naučni rad-

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Rezime

U radu je predložena spektrofluorimetrijska metoda za određivanje sadržaja hesperidina u sokovima od pomorandže zasnovana na sposobnosti fluorescencije kompleksa aluminijum – hesperidin. Utvrđena je oblast linearnosti za određivanje hesperidina u metanolno-vodenim rastvora od 0.08 do 18.0 μg/mL, pri čemu je LOD= 0.023 μg/mL i LOQ= 0.070 μg/mL, a recovery vrednost 97.8 – 99.7 %. Metoda je pojednostavljena izostavljanjem često korišćenih površinski aktivnih materija koje se koriste u sličnim procedurama i uspešno je primenjena za određivanje sadržaja hesperidina u sokovima od pomorandže prisutnim na tržištu Srbije.

Ključne reči: hesperidin, sok od pomoraandže, spektrofluorometrija.