



## Analysis of the components of pumpkin seed oil in suppositories and the possibility of its use in pharmaceuticals

Vorobyova O. A.<sup>a</sup>, Bolshakova A. E.<sup>a</sup>, Pegova R. A.<sup>a</sup>, Kol'chik O. V.<sup>b</sup>, Klabukova I. N.<sup>a</sup>,  
Krasilnikova E. V.<sup>c</sup> and Melnikova N. B.<sup>a</sup>

<sup>a</sup>Department of Pharmaceutical Chemistry, Nizhny Novgorod State Medical Academy, Russian Federation, Nizhny Novgorod, Minin sq., 10/1

<sup>b</sup>Department of Ophthalmology, Nizhny Novgorod Regional Clinical Hospital. Semashko, Russian Federation, Nizhny Novgorod, Rodionova str., 190

<sup>c</sup>Department of Organic and Inorganic Chemistry, Nizhny Novgorod State Medical Academy, Russian Federation, Nizhny Novgorod, Minin sq., 10/1

---

### ABSTRACT

The procedures of RP-HPLC-analysis of the tocopherols mixture and  $\beta$ -sitosterol and UV-Vis-analysis of carotenoids of Pumpkin - *Cucurbita pepo* - seed oil (25 %) in the presence of thymol (5 %) in the fat-based suppositories have been developed. Results of determination of  $\gamma$ - and  $\alpha$ -tocopherols,  $\beta$ -sitosterol in suppositories was carried out by RP-HPLC method according to United States Pharmacopeia with the aspect of linearity, accuracy and repeatability. It has been shown that the Pumpkin seed oil contains 6 mg %  $\alpha$ -tocopherol, about 50 mg %  $\gamma$ -tocopherol and  $\gamma$ -tocotrienol mixture, 105-150 mg %  $\beta$ -sitosterol and 0,6 - 1,0 mg % carotenoids. Lutein and zeaxanthin in a ratio of (60:40) as the main carotenoids and cryptoxanthin, violaxanthin and other hydroxy- and epoxy-carotenoids as the minor components in the oil have been found. Accordingly, one suppository contains 240  $\mu$ g tocopherols, 30-50  $\mu$ g carotenoids and 630  $\mu$ g  $\beta$ -sitosterol. The high content of  $\beta$ -sitosterol, thymol,  $\gamma$ -tocopherol and  $\gamma$ -tocotrienol mixture, as well as carotenoids allows the use of the medicine "Bioprost<sup>®</sup>" and Pumpkin seed oil as a pharmaceutical substance in other dosage forms for the treatment of lipid-associated diseases, benign prostatic hyperplasia and malignant neoplasms type myeloma.

**Key words:** Pumpkin seed oil, suppositories, HPLC-analysis, tocopherols,  $\beta$ -sitosterol.

---

### INTRODUCTION

Natural vegetable oils from various species and subspecies of Pumpkin (about 700) are the great interest for pharmacy and medicine [1]. It has been considered, that hypolipidemic and hepatoprotective activity due to a high content of linoleic and oleic acids,  $\Delta^7$ - and  $\Delta^5$ -sterols, whose the main representative is  $\beta$ -sitosterol [2]. (Fig. 1)

The high concentration of unsaturated fatty acids (80%) together with phytosterols opens up the possibility for the treatment of lipid-associated diseases such as atherosclerosis [3, 4]. Assumed that synthesized in the body or dietary cholesterol is absorbed into the blood and into the micelles of low density lipoproteins (LDL) up to disposal by cells [3]. Hypocholesterolemic effect of  $\beta$ -sitosterol has been caused by the principle of antimetabolites image due to the chemical structure of cholesterol by displacing it from LDL in plasma [5]. The other mechanism of hypocholesterolemic action has been proposed as the formation of stable complexes with  $\beta$ -sitosterol, which is absorbed from the gastrointestinal tract much worse than cholesterol [6, 7].

The important application of pumpkin seed oil is its use in the treatment of benign prostatic hyperplasia and other prostate diseases, including the treatment of symptomatic urinary disorders [8]. The pumpkin oil in the form of injections (Depostat injection) has been recommended by European medicine (The International Prostate Symptom



## EXPERIMENTAL SECTION

**Materials and Reagents.** Standard substances: ( $\pm$ )  $\alpha$ -tocopherol (Supelco, 47783), rac- $\beta$ -tocopherol (50 mg/ml in hexane, Supelco, 46401-U),  $\gamma$ -tocopherol (Supelco, 47785),  $\delta$ -tocopherol (Supelco, 47784),  $\alpha$ -tocotrienol (Fluka, 07205),  $\beta$ -tocotrienol (Fluka, 05644),  $\gamma$ -tocotrienol (Fluka, 49634),  $\delta$ -tocotrienol (Fluka, 69745), zeaxanthin (Fluka, 14681),  $\alpha$ -carotene (Fluka, 50887),  $\beta$ -carotene (Sigma, 22040),  $\beta$ -sitosterol 95%, pumpkin seed oil (SAF 42-8110-06). **Reagents:** chemically pure grade KOH, nitrogen, MgO, Al<sub>2</sub>O<sub>3</sub>, CaCO<sub>3</sub>, SiO<sub>2</sub>, CaO. **Solvents:** ethanol, hexane, chloroform, petroleum ether, ethyl acetate. **Solvents (HPLC grade)** acetonitrile, methanol, dichloromethane.

**The composition of the commercial formulation** Rectal suppositories "Bioprost<sup>®</sup>" (JSC «Intelpharm", Nizhny Novgorod, Russia), g: Pumpkin Seed Oil - 0.5, thymol - 0.1; Witepsol H-15, W-35 (1:1) - 1.4.

**Preparation of model mixtures.** *Standard solution of pumpkin seed oil* 1(0.25 g), 2(0.5 g), 3(0.75 g): the standard substance of pumpkin seed oil was placed in a 50 mL glass and thymol (0.1 g), Witepsol W35 (0.7 g) and Witepsol H15 (0.7 g) were added. The mixture was heated by water bath at 50°C until meltdown. Hexane (15 mL) was added into the glass and was stirred. The solution was poured into a 50 mL volumetric flask. The glass was washed 3 times with 10 mL hexane; each portion was poured into the volumetric flask. The solution in the flask was brought to the mark with hexane (corresponds to 50 %, 100 %, 150 % of the expected concentration of the test solution). *Test solution of pumpkin seed oil:* One suppository was placed in a 50 mL glass and was heated by water bath at 50°C until meltdown. Hexane (15 mL) was added into the glass and was stirred. The solution was poured into a 50 mL volumetric flask. The glass was washed 3 times with 10 mL hexane; each portion was poured into the volumetric flask. The solution in the flask brought to the mark with hexane. *Standard solution of 1,2,3 tocopherols.* 2.8 g of base (Witepsol grade H-15 and W-35 = 1:1) was melted at 50-60°C by water bath, 0.2 g of thymol and 1.3 mL (2.5 mL, 5 mL)  $\alpha$ -tocopherol standard solution (5 mg, 10 mg, 20 mg), ascorbic acid (0.02 g), 96 % ethanol (50 mL) were added. Standard solutions were saponified and purified from carotenoids by the procedures described below, and the dry residues were dissolved in 1 mL of eluent (50:44:6 methanol / acetonitrile / dichloromethane). *Test solution tocopherols:* 2 suppositories were melted at 50-60°C by water bath, then ascorbic acid (0.02 g), 96 % ethanol (50 mL) were added. Standard solutions were saponified and purified from carotenoids by the procedures described below, and the dry residues were dissolved in 1 mL of eluent (50:44:6 methanol / acetonitrile / dichloromethane).

**Devices.** UV spectrophotometer Specord S100 Bioline (Analytic Jena, Germany); HPL chromatograph «LC-20 Prominace» (Shimadzu, Japan) in reversed-phase mode. Analytical Column - 4.6 mm x 25 cm, particle size 5 micrometers, C18 (Supelco Discovery<sup>®</sup> C18). Nozzle for filtration of samples (Non-Sterile 4 mm Millex<sup>®</sup> Syringe Filter Unit) with a pore size no greater than 0.45  $\mu$ m.

**Saponification.** Ascorbic acid (0.02 g), 96 % ethanol (50 mL) and two suppositories (4.0000 $\pm$ 0.0002) were added and the mixture was heated at 40°C by water bath until homogenization. The mixture was heated at 70°C, then it was incubated in the presence of 30 mL of 60 wt. % aqueous KOH solution for 30 minutes. 100 mL of water was added into two-phase medium, during which reaction mixture was become homogeneous. Unsaponifiable components of the reaction mixture were extracted with two portions of hexane (100 mL each). The combined hexane fractions were treated by 100 mL of 1 % ascorbic acid solution, then twice by 100 mL of water, consequently; then hexane solution was dried by Na<sub>2</sub>SO<sub>4</sub>. At the last stage hexane was removed under nitrogen up to 10-15 mL.

**Purification from carotenoids.** Hexane solution was passed through a 10 – cm - high column (30 – cm - long, 1-1.5 – cm - dia) with a layer of MgO (or CaCO<sub>3</sub>, Al<sub>2</sub>O<sub>3</sub>); then the column was rinsed twice with 10-15 mL of hexane and hexane was removed under nitrogen.

**RP-HPLC-analysis.** **A. Analysis of tocols.** Mobile phase: 50:44:6 (v / v / v) methanol / acetonitrile / dichloromethane, UV detection (284 nm and 295 nm) at 30°C. Before analysis the sample solution was filtered. Flow rate of mobile phase was equal to 1 mL / min. HPLC analysis was carried out using test standard solutions. **B. Analysis of  $\beta$ -sitosterol.** Mobile phase: 85:15 (v / v) acetonitrile / ethanol 95%, and UV detection (210 nm) at 40°C. Statistical treatment was performed using Statistica 7.0.

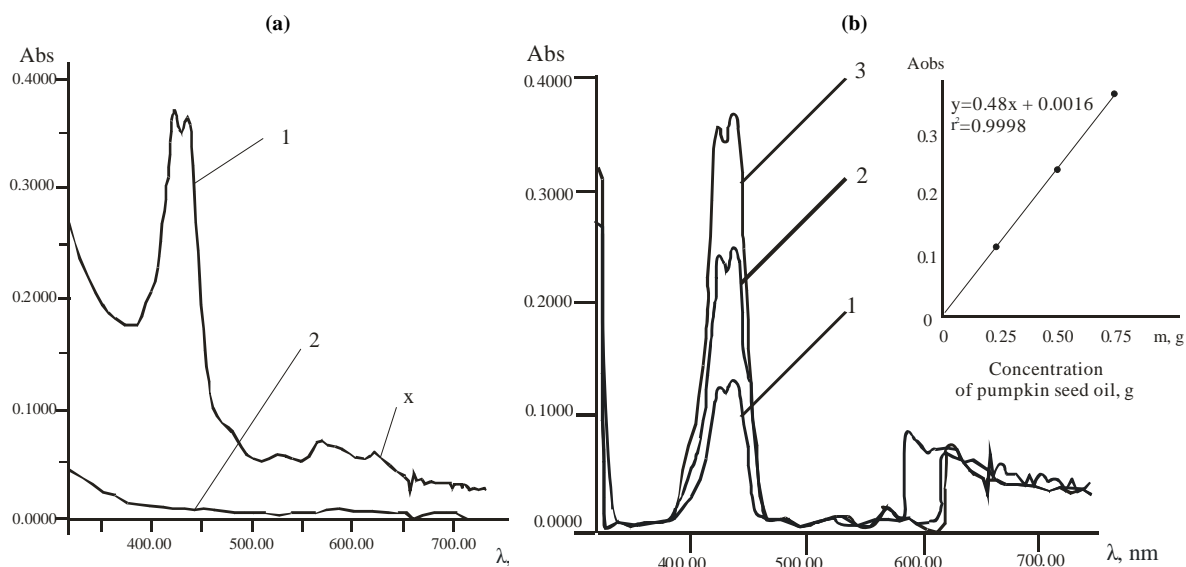
## RESULTS AND DISCUSSION

**1.1. UV-Vis-analysis of the carotenoids in suppositories**

Polar sorbents such as CaCO<sub>3</sub>, MgO, Al<sub>2</sub>O<sub>3</sub> were used for adsorption of carotenoids from hexane solution of "Bioprost<sup>®</sup>" suppository for UV-Vis analysis. Adsorption capacity of sorbents was increased in the series:

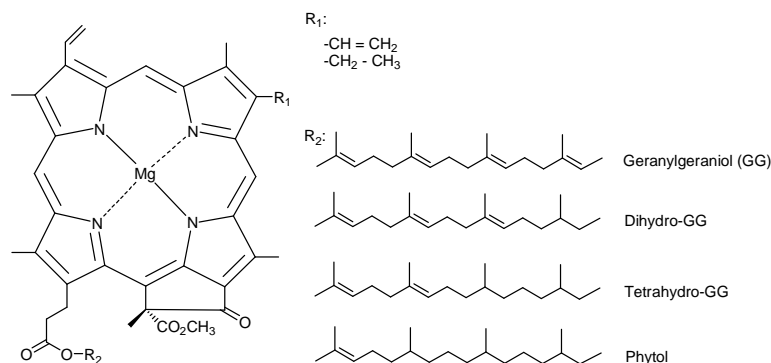
MgO > Al<sub>2</sub>O<sub>3</sub> > CaCO<sub>3</sub> (1)

The shape and position of the absorption bands in UV-Vis spectra were the same for all hexane solutions after adsorption, but the optical density in the visible region of carotenoids (400-500 nm) increased in the same row (1). Figure 3a shows a typical visible spectrum of a pumpkin seed oil hexane solution after passing it through a column with MgO, where bands have  $\lambda_{\max}$  equal to 424 and 434 nm. The same absorption bands were observed if carotenoids were desorbed from the column by chloroform, but the optical density had different values at  $\lambda_{\max} = 424$  and 434 nm. Thus, it may be possible to analyze the content of carotenoids in hexane or chloroform solution using appropriate calibration curves for each solvent.



**Fig. 3. Visible spectra of hexane solutions of carotenoids in pumpkin seed oil and suppositories: a) 1 - extract of pumpkin seed oil in hexane, 2 - hexane solution of thymol and Witepsol, b) solutions of model composition 1,2,3: fat base (70%), thymol (5%), pumpkin seed oil [12.5% (1), 25% (2) and 50% (3), respectively]. The spectra are shown considering base line. Inset: the dependence of the optical density on the content of pumpkin seed oil in model compositions 1, 2, 3**

Besides, the shoulder in the region of 474 nm and the bands of 531 nm, 572 nm, and 630 nm, characteristic for protophilic compounds, e.g., chlorophyll (Figure 4) [2], were observed in the spectra of hexane extracts after saponification.



**Fig. 4. Formula of chlorophyll**

The structures of carotenoids were investigated according to data of well-known UV-Vis spectra [14]. Carotenoids of vegetable oils occur only in stable form where all double bonds are in trans configuration (“all-trans-form”). The general formula of the major carotenoids of pumpkin oils are presented in Table 1.

We assumed that pumpkin seed oil contains hydroxyl- and epoxy- carotene derivatives (Figure 4), because they were absorbed by chloroform or tetrahydrofuran much better than by hexane from the adsorption column, and absorption spectra characteristics are similar to these derivatives.

The structure and composition of carotenoids are determined either by the processes of biosynthesis and biotransformation into oil by the scheme:

**$\alpha$ -series:**  $\alpha$ -carotene  $\rightarrow$   $\alpha$ -cryptoxanthin  $\rightleftharpoons$  lutein  $\rightleftharpoons$  luteoxanthin

**$\beta$ -series:**  $\beta$ -carotene  $\rightarrow$   $\beta$ -cryptoxanthin  $\rightleftharpoons$  zeaxanthin  $\rightleftharpoons$  violaxanthin

or incorrect oil storage [1].

**Table 1** The main carotenoids of *Cucurbita pepo* and *Cucurbita maxima* pumpkins [15]

Carotenoid			$\lambda_{\max}$ , nm	$E_{1cm}^{1\%}$
	R <sub>1</sub>	R <sub>2</sub>		
$\alpha$ -carotene			422, 445	2710
$\beta$ -carotene			425, 450	2592
$\alpha$ -cryptoxanthin			428, 450	2460
$\beta$ -cryptoxanthin			-	-
lutein			421, 445	2550
zeaxanthin			428, 450	2480
luteoxanthin			-	-
violaxanthin			440	2550

The oxidation and cleavage of the epoxy groups of carotenoids are reversible and allow regenerating the initial compounds. Violaxanthin contains epoxy groups at both ends of a molecule and photoreduction in the presence of ascorbic acid leads to formation of zeaxanthin (violaxanthin's cycle).

The absorption spectrum of the hydroxy and epoxy derivatives of  $\alpha$ -carotene, such as lutein having eleven double bonds are identical to spectrum of  $\alpha$ -carotene, considering that the polyene chromophore system is the same, and hydroxy or epoxy groups do not influence  $\lambda_{\max}$ . Accordingly, UV-Vis spectra of  $\beta$ -carotene, zeaxanthin and violaxanthin are identical. Specific extinction coefficients  $E_{1cm}^{1\%}$  of hydroxy- and epoxy- carotene derivatives have also similar values. Consequently, the use of the optical density of bands at  $\lambda_{\max 1} = 424$  nm and  $\lambda_{\max 2} = 434$  nm in solutions of pumpkin seed oil and of suppositories – considering the calibration curve data (Figure 4b, inset) of the dependence of optical density on the concentration of  $\alpha$ -standards and  $\beta$ -carotene – allows to calculate the total carotenoid content in the oil and suppositories for the model mixture (Figure 4b), assuming the average specific extinction coefficient equal to 2510.

The total carotenoids content (mg) of one suppository has been calculated by the formula:

$$m, \text{mg} = \frac{(A_{\text{obs.}} - b) \cdot V(\text{mL})}{E_{1cm}^{1\%}} \cdot C^o (\text{mg} \cdot \text{mL}^{-1}) \quad (2),$$

where  $A_{\text{obs}}$  – optical density of the test hexane solution of suppository;  $b$  – the correction coefficient of the optical density relative to baseline;  $V$  – volume of solution (mL);  $E_{1\text{cm}}^{1\%}$  – specific extinction coefficient equal to  $\beta$ ,  $\beta$ -carotene-3, 3'-diol in hexane - 2480 and  $\alpha$ ,  $\beta$ -carotene-3, 3'-diol in hexane - 2550;  $C^0$  – concentration of solution  $10 \text{ mg mL}^{-1}$ .

The total content of carotenoids in one suppository, calculated according to the formula (2), is  $48.0 \pm 2.0 \mu\text{g}$ .

A good **linearity** was achieved in the concentration ranges of  $1 \mu\text{g mL}^{-1}$  –  $30 \mu\text{g mL}^{-1}$  for carotenoids. The regression equations and correlation coefficient for the reference were  $y = 0,48x + 0,0016$ ,  $r^2 = 0,9998$ . The experiment was performed three times and the value was used for the calculations. The data were analyzed by linear regression least squares fitting. The statistical data obtained are given in Tables 2, 3.

**Accuracy** was established by measuring the amount of the quantitative content of carotenoids in the solutions obtained by adding a certain amount of standard to the test solution. The acceptance criterion was the average percent recovery using working solutions of carotenoids over the concentration range  $24\text{--}100 \mu\text{g mL}^{-1}$ . The results of Table 3 indicated that UV-Vis method of analysis of carotenoids in the suppositories were accurate, because average recovery percent must be within  $100 \pm 5 \%$ .

**Table 2** Repeatability results for determination of total carotenoids

Data	Repeatability								
	1	2	3	4	5	6	7	8	9
carotenoids ( $\mu\text{g}$ )	24	24,3	23,8	47,9	48,1	47,5	96,4	95,6	95,8
Mean	24,0			47,8			95,9		
RSD (%)	1,06			0,64			0,44		

**Table 3** Accuracy results [Recovery (%)] for determination of total carotenoids

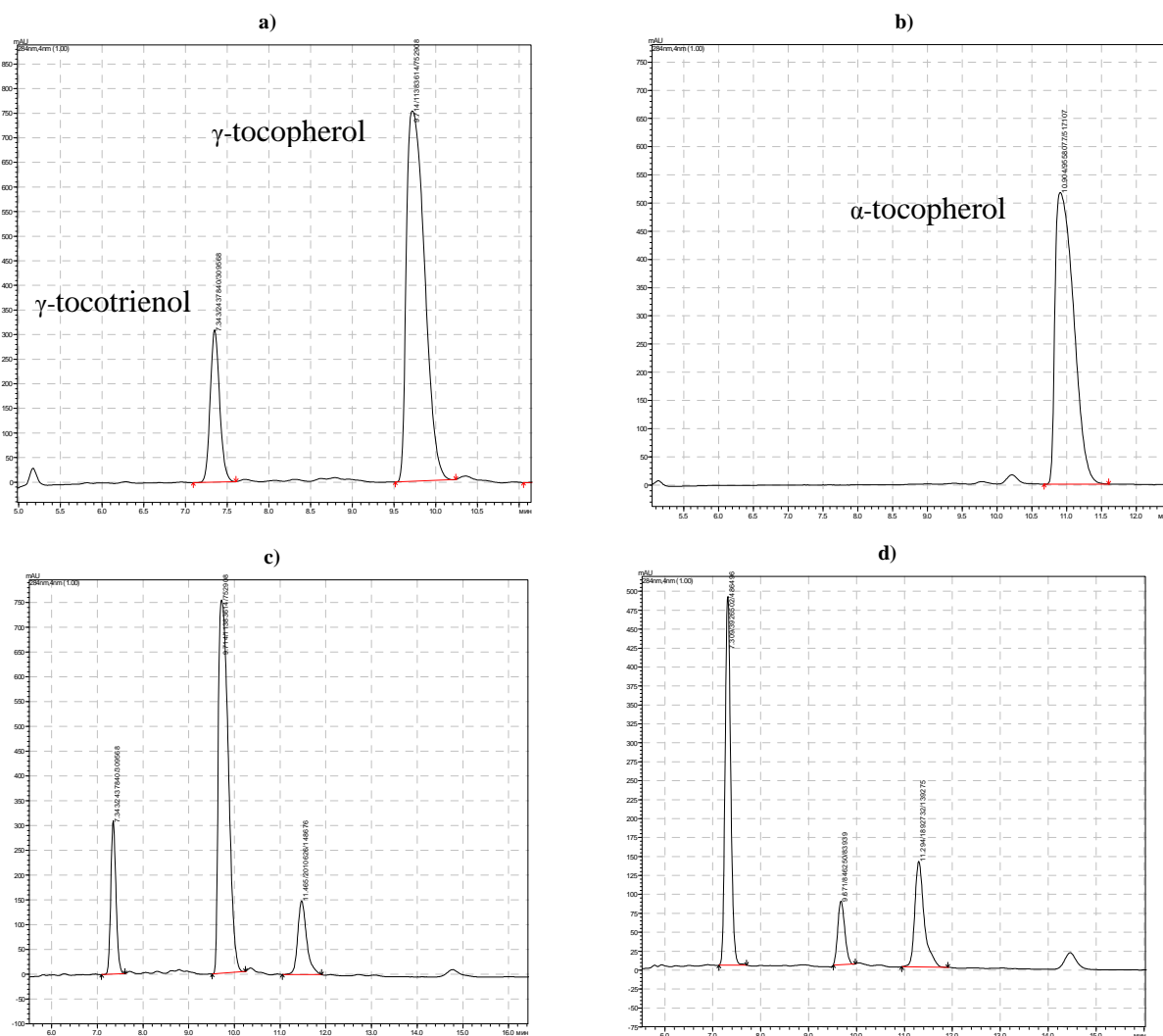
Total content of $\beta$ , $\beta$ -carotenoids ( $\mu\text{g}$ )	Added carotene ( $\mu\text{g}$ )	Calculated content ( $\mu\text{g}$ )	Found content ( $\mu\text{g}$ )	Recovery (%)
24	10	34	32,3	95
24	10	34	31,8	93,5
24	10	34	33,4	98,2
48	20	68	67,8	99,7
48	20	68	65,3	96,0
48	20	68	66,4	97,7
96	30	126	125,7	99,8
96	30	126	126,1	100,1
96	30	126	126,5	100,4
Mean – 97,8%				

## 1.2. RP-HPLC - content analysis of tocopherols and $\beta$ -sitosterol in suppositories and pumpkin seed oil

At the first stage pumpkin seed oil or suppository was treated by alcohol solution of potassium hydroxide in presence of ascorbic acid under nitrogen both for the fat saponification and hydrolytic cleavage of carotenoids oxy-derivates, esterified by fatty alcohols (palmitic, stearic, etc.). Carotenoids oxy-derivates and chlorophyll, which are present in pumpkin seed oil, were removed using adsorption column with polar sorbents as described above. The dry residue was dissolved in one mL of the eluent after hexane removal under nitrogen.

In this work RP-HPLC method of analysis of tocopherol mixture, recommended in paper [14] for assay of tocopherols in brown rice, was used: isocratic profile, temperature  $30^\circ\text{C}$ , eluent: 44:50:6, v / v / v (acetonitrile / methanol / dichloromethane), flow rate  $1.0 \text{ mL min}^{-1}$ , UV detector (284 nm).

It has been shown that ratio  $\gamma$ -tocotrienol and  $\gamma$ -tocopherol was varied from 1:2 to 2:1 by increasing process time from 30 minutes to 1 hour and it is necessary to use more ascorbic acid than it is given in the known methods, as well as to control the time of saponification. Figure 5 shows the HPL-chromatograms of model suppository solutions after various time of saponification (5a, b) and standard samples of  $\gamma$ -tocotrienol,  $\alpha$ - and  $\gamma$ -tocopherols (5c,d).



saponification time - 30 minutes

saponification time 1 hour

Name	Retention time	Peak area				Peak height			
		30 min	Ratio	1 hour	Ratio	30 min	Ratio	1 hour	Ratio
$\alpha$ -tocopherol	11,47	2010626	1,0	1892732	2,2	148676	1,0	139275	1,7
$\gamma$ -tocopherol	9,71	11383614	5,7	846250	1,0	752908	5,1	83939	1,0
$\gamma$ -tocotrienol	7,34	2437840	1,2	3926950	4,6	309568	2,1	486496	5,8

Fig. 5. HPL-chromatograms: conditions of sample preparation of standards (saponification 30 min) a)  $\gamma$ -tocopherol and  $\gamma$ -tocotrienol, b) 95%  $\alpha$ -tocopherol and 5%  $\alpha$ -tocotrienol. Eluent - 44:50:6, v / v / v (acetonitrile / methanol / dichloromethane), flow rate 1.0 mL min<sup>-1</sup>, UV detector (284 nm) 30°C. HPL-chromatograms of model suppository solutions after various time of saponification: c) 30 minutes, d) 1 hour

$\beta$ -sitosterol in pumpkin seed oil – *Cucurbita pepo* and *Cucurbita maxima* species – and in suppositories was analyzed by another procedure of RP-HPLC- analysis using the same samples after saponification and  $\beta$ - sitosterol as standards. Eluent was chosen as a mixture of solvents – acetonitrile / 96% ethyl alcohol (85/15), column temperature: 40 °C, the wavelength of UV-detector - 210 nm according to work [16]. Taking into account that the minor components – campesterol and stigmasterol – weren't medicines for treatment benign prostatic hyperplasia and other prostate diseases, including the treatment of symptomatic urinary disorders, quantitative determination of these sterols wasn't carried out in this paper.

HPL- chromatograms of  $\beta$ - sitosterol and the sample of pumpkin seed oil after saponification are shown in Figure 6. Standard of  $\beta$ -sitosterol contains brassicasterol as an impurity (peak 1). Apparently campesterol was an impurity that corresponded peak 3, and peak 4 corresponded  $\beta$ -D-glucopyranosid form of  $\beta$ -sitosterol.

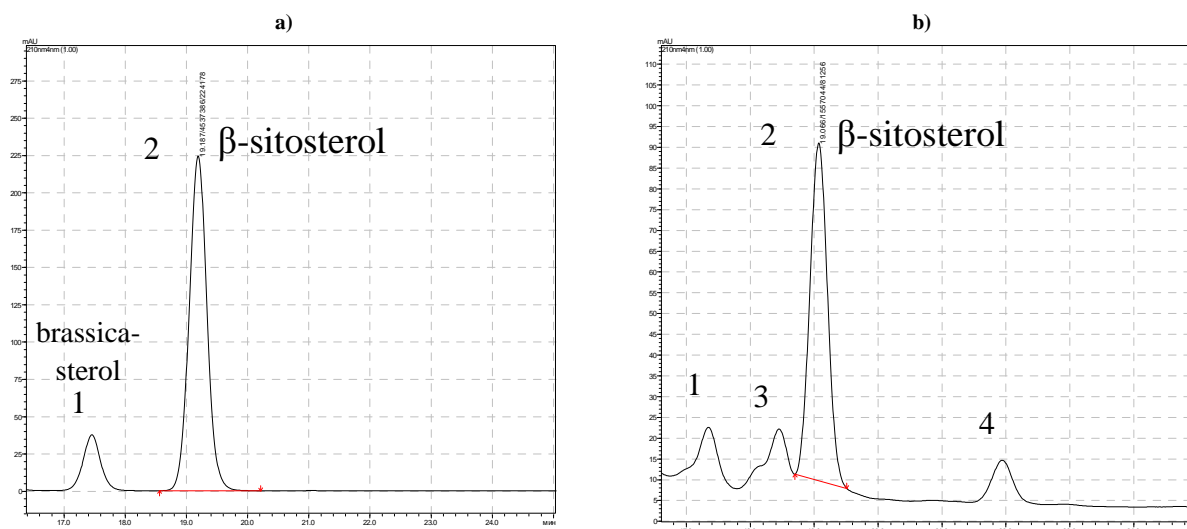


Fig. 6. HPL-chromatograms of samples after saponification: a)  $\beta$ -sitosterol, b) pumpkin seed oil. Eluent - 85:15, v / v (acetonitrile / 96% ethyl alcohol), flow rate 1.0 mL / min, UV detector (210 nm) 40°C

The proposed RP-HPL method was examined with the aspect of linearity and range, accuracy, precision, according to the United States Pharmacopeia [17].

System suitability was determined by six replicate injections of the system suitability solution. The acceptance criteria were less than 2% relative standard deviation (RSD) for peak areas, greater than 174000 column plates, less than 1.5 of the USP tailing factor, and greater than 1.5 of the resolution. The results obtained were all within the acceptable limits. The resolutions among  $\gamma$ - and  $\alpha$ -tocopherols and the closest eluting peaks were bigger than 2 which indicated that this method was reliable for the quantification of  $\gamma$ - and  $\alpha$ -tocopherols. A typical chromatogram for the system suitability test is shown in Figure 5. The same result has been shown for  $\beta$ -sitosterol and the impurities (Figure 6).

**Linearity** was checked by analyzing 9 working solutions of  $\gamma$ - and  $\alpha$ -tocopherols over the concentration range: 0.01-0.20 mg mL<sup>-1</sup> (0.01, 0.02, 0.04, 0.08, 0.10, 0.20 mg mL<sup>-1</sup>) and 0.015-0.48 mg mL<sup>-1</sup> (0.015, 0.030, 0.060, 0.120, 0.240, 0.480 mg mL<sup>-1</sup>) for  $\beta$ -sitosterol. The following results were obtained:  $y=82104.0 x$ ,  $r^2=0.982$  for  $\alpha$ -tocopherol;  $y=11383614.0 x$ ,  $r^2=0.999$  for  $\gamma$ -tocopherol and  $y=1763502.7 x$ ,  $r^2=0.990$  for  $\beta$ -sitosterol, where  $y$ =peak area,  $x$ =concentration of solution,  $r^2$ =the square of determined correlation coefficient. The results indicated that the method was linear over the concentration range studied.

**Accuracy** of the method was assessed by recovery test. A known amount of each standard (pumpkin seed oil,  $\gamma$ - and  $\alpha$ -tocopherols,  $\beta$ -sitosterol) was added to blank sample composed of all the excipients (Witepsol, thymol) equivalent to the ratio of the suppository formulation, which was then mixed, saponificated, extracted and were made other operations described above for RP-HPLC analysis; then, subsequently diluted to yield three different concentrations for pharmaceutical substances. These samples were prepared as described in the experimental part and analyzed as previously described. The corresponding percentage recovery data are summarized in Table 4.

Table 4 Accuracy results [Recovery (%)] for the determination of  $\alpha$ ,  $\gamma$ - tocopherols and  $\beta$ - sitosterol

Standard	Level	Reanalyzed sample in ( $\mu\text{g mL}^{-1}$ )	Amount of std added to reanalyzed sample in ( $\mu\text{g mL}^{-1}$ )	Total amount of std found in ( $\mu\text{g mL}^{-1}$ )	SD	RSD (%) (n=7)	Recovery (%)
$\alpha$ -tocopherol	0	0,06	0	0,058	0,11	1,15	96,7
	50%	0,06	0,03	0,086	0,08	0,47	95,6
	100%	0,06	0,06	0,123	0,07	0,35	102,5
Average recovery							98,3
$\gamma$ -tocopherol	0	1,0	0	0,980	0,13	0,45	98,0
	50%	1,0	0,5	1,487	0,09	0,38	99,1
	100%	1,0	1,0	1,989	0,10	0,27	99,5
Average recovery							98,9
$\beta$ - sitosterol	0	1,5	0	1,470	0,12	0,48	98,0
	50%	1,5	0,75	2,230	0,07	0,37	99,0
	100%	1,5	1,5	3,020	0,11	0,29	100,7
Average recovery							99,2

\*Mean of three determinations for each concentration



**Repeatability** or intra-day precision was investigated by injecting six replicate sample solutions on the same day. Inter-day precision was assessed by analyzing newly prepared sample solutions in triplicate over three consecutive days. Precision was expressed as RSD value of the analytic peaks. RSD values obtained for the peak areas of  $\gamma$ - and  $\alpha$ -tocopherols,  $\beta$ -sitosterol of a single day (day 1, n=6) were 0.45%, 0.51% and 0.56%, respectively. RSD values on triplicate injections on three successive days (days 1–3, n=9) were 1.5% and 1.9%, respectively. The results implied that the method developed was accurate for the determination.

#### Assay of $\gamma$ - and $\alpha$ -tocopherols, $\beta$ -sitosterol in pumpkin seed oil and suppositories

The substances – pumpkin seed oil,  $\gamma$ - and  $\alpha$ -tocopherols,  $\beta$ -sitosterol of suppository – were analyzed using the developed procedures. The obtained satisfactory percentage results, found for  $\gamma$ - and  $\alpha$ -tocopherols,  $\beta$ -sitosterol, were in a good agreement with the literature data for pumpkin seed oil – *Cucurbita pepo* and *Cucurbita maxima* species. These results correspond to theoretical calculated concentration in formulation of dosage form correlated to pumpkin seed oil content. Unfortunately,  $\gamma$ - tocopherol and  $\beta$ -sitosterol are not designated in the medical instruction and label claimed.

It has been shown that the Pumpkin seed oil contains 6 mg %  $\alpha$ -tocopherol, about 50 mg %  $\gamma$ -tocopherol and  $\gamma$ -tocotrienol mixture, 105-150 mg %  $\beta$ -sitosterol. Accordingly, one suppository contains 240  $\mu$ g tocols and 630 $\pm$ 20  $\mu$ g  $\beta$ -sitosterol (Table 5).

Table 5 Assay results of  $\gamma$ - and  $\alpha$ -tocopherols,  $\beta$ -sitosterol in "Bioprost<sup>®</sup>" suppository

Batch number	Added pumpkin seed oil (mg/suppository)	Found in suppository ( $\mu$ g)			RSD (%)		
		tocopherol		$\beta$ -sitosterol	tocopherol		$\beta$ -sitosterol
		$\gamma$ -	$\alpha$ -		$\gamma$ -	$\alpha$ -	
N1	0.48	212.2	24.4	625	0.88	2.54	0.48
N2	0.50	214.1	26.2	630	0.94	1.68	0.49
N3	0.52	215.3	27.3	636	1.06	1.72	0.51

N1, N2 and N3 refer to three different batches.

## CONCLUSION

Thus, procedures of RP-HPLC- analysis of the tocols mixture and  $\beta$ - sitosterol as well as UV-Vis- analysis of carotenoids in pumpkin seed oil – *Cucurbita pepo* – and in the fat-based suppositories containing pumpkin seed oil (25%) in presence of thymol (5 %) have been developed. Results of the determination of  $\gamma$ - and  $\alpha$ -tocopherols,  $\beta$ -sitosterol in suppositories were carried out by RP-HPLC method according to Russian Pharmacopoeia 2010 with the aspect of linearity, accuracy and repeatability.

It has been shown that the original pumpkin seed oil contains 6 mg%  $\alpha$ - tocopherol, about 50 mg% mixture  $\gamma$ -tocopherol and  $\gamma$ - tocotrienol, 105-150 mg%  $\beta$ - sitosterol, and 0.6-1.0 mg% carotenoids. Lutein and zeaxanthin in a ratio of (60:40) are the main components of the oil, and the other hydroxy - and epoxy- carotenoids – cryptoxanthin, violaxanthin – are the minor ones.

The found concentration of  $\beta$ -sitosterol is 630  $\mu$ g in a suppository, which is required for treatment of benign prostate hyperplasia, as well as of lipid-associated diseases like atherosclerosis.

It is very important that  $\gamma$ -tocopherol and  $\gamma$ -tocotrienol are the main components of pumpkin seed oil, because their pharmacological activity is more effective for such diseases as cardioprotective [18-20], hypolipidemic [18-20], hepatoprotective [21], anti-inflammatory [22-24], antitumor [25-31], neuroprotective activity [32-34], but hemolysis is significantly lower [31] than that for  $\alpha$ -tocopherol. At the same time it is necessary to indicate that mixed tocopherols and tocotrienols are bioavailable, although tocotrienols have shorter plasma half-lives and, probably, different tissue distribution than  $\alpha$ - tocopherol [35].

The presence of carotenoids - provitamin A is also significant for the dosage form with respect to protective antioxidant function of tocols.

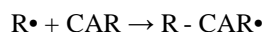
Antioxidant activity of carotenoids is mainly determined as ability to physically capture singlet oxygen:



After that carotenoids in the triplet state can easily return to original state (CAR), giving off energy as heat. In this reaction, carotenoids may additionally take hold of singlet oxygen  ${}^1\text{O}_2$ . The efficiency of capture of the carotenoids is increased, and the energy of their excited state is decreased with the number of conjugated double bonds in the molecule, wherein the epoxy group can increase the capture ability. Carotenoid triplet state is so a low-energy state

that it is unable to generate other reactive particles by energy transfer; instead energy dissipation they produce heat into the environment. Thus, carotenoids react as catalysts of singlet oxygen deactivation.

A second important antioxidant activity of CAR is the interaction with the radicals which break off hydrogen from the carotenoid (4), and an unpaired electron moves to the carotenoid (5) or forms an adduct with the radical.



The capture of radical particles depends on formation of adducts with resonance stabilized centers that inhibit lipid peroxidation, stopping the chain reaction. This chemical reaction leads to the degradation of carotenoids and is accompanied by loss of colour. Accordingly, the antioxidant activity of carotenoids is more effective than  $\alpha$ -tocopherol in consequence of several antioxidant-action mechanisms [36-38].

Besides, the dosage form contains thymol, which can act as antioxidant in the lipid peroxidation process; also it has an inhibitory effect in the treatment of various kinds of cancer, antiinflammatory and antiseptic activity, thereby increasing the pharmacological effects of the medicines.

Thus, taking in account the pharmacological effects of active ingredients, such as  $\beta$ - sitosterol,  $\gamma$ - tococls and carotenoids, detected by us and which were not included in the formulation of "Bioprost<sup>®</sup>" dosage form, synergism of tococls and carotenoids action, we would like to propose to use this dosage form for treatment of other diseases, not only for benign prostate hyperplasia.

## REFERENCES

- [1] CA Howitt; BJ Pogson, *Plant, Cell and Environment*, **2006**, 29, 435–445.
- [2] GO Fruhwirth; A Hermetter, *Eur. J. Lipid Sci. Technol.*, **2007**, 109(11), 1128–1140.
- [3] I Landekaa; D Đikićb; I Radišića et al., *Croatian Journal of Food Technology, Biotechnology and Nutrition*, **2011**, 6 (1-2), 63-68.
- [4] MF Ramadan; R Zayed; M Abozid and MMS Asker, *Grasas y aceites*, **2011**, 62 (4), 443-452.
- [5] BA Griffin; SC Cunnane, *Introduction to human nutrition*, **2009**, 2, 86-121.
- [6] KF Klippel; DM Hiltl; B Schipp, *Br. J. Urol.*, **1997**, 80(3), 427-432.
- [7] SB Strum; W Gland, *Life Extention Foundation*, **2005**, 57-61.
- [8] A Shrivastava; VB Gupta, *J. Midlife Health*, **2012**, 3(1), 10–19.
- [9] H Satooka; I Kubo, *J. Agric Food Chem.*, **2012**, 60(10), 2746-2752.
- [10] DD Deb; G Parimala; SS Devi; T Chakraborty, *Chem. Biol. Interact.*, **2011**, 193(1), 97-106.
- [11] SS Hsu; KL Lin; CT Chou; AJ Chiang et al., *Eur. J. Pharmacol.*, **2011**, 670(1), 85-91.
- [12] HT Chang; SS Hsu et al., *Pharmacology*, **2011**, 88(3-4), 201-212.
- [13] V Velasco; P Williams, *Chilean JAR*, **2011**, 71(2), 313-322.
- [14] AP De Leenheer; WE Lambert; JF Van Bocxlaer. *Modern Chromatographic Analysis of Vitamins*, Marcel Dekker Inc., 1<sup>st</sup> Edition, New York, **2000**; 183-185.
- [15] M Kreck; P Kurbel et al., *Journal of Applied Botany and Food Quality*, **2006**, 80, 93-99.
- [16] JC Ye; WC Chang et al., *Journal of Medicinal Plants Research*, **1990**, 67(11), 827-831.
- [17] United States Pharmacopoeia, XXII, **1990**, 1225.
- [18] AC Tomeo; M Geller et al., *Lipids*, **1995**, 30(12), 1179-1183.
- [19] DK Kooyenga; TR Watkins; M Geller; M. Bierenbaum, *Micronutrients and health*, **2001**, 366- 375.
- [20] AHG Rasool; ARA Rahman; KH Yuen; AR Wong, *Archives of Pharmacal Research*, **2008**, 31(9), 1212–1217.
- [21] AJ Sanyal; N Chalasani; KV Kowdley et al., *N. Engl. J. Med.*, **2010**, 362(18), 1675-1685.
- [22] BB Aggarwal; KS Ahn. Targeting Inflammation for Prevention and Treatment of Cancer by Tocotrienols: Food for Thought, *PIPOC*. **2007**, 7-20.
- [23] SJ Wu; PL Liu; LT Ng, *Mol. Nutr. Food Res.*, **2009**, 52(8), 921–929.
- [24] G Tahan; E Aytac et al., *Canadian J. Surgery*, **2011**, 54(5), 333–338.
- [25] CK Sen; S Khanna; S Roy, *Life Sci.*, **2006**, 78(18), 2088-2098.
- [26] C Rink; G Christoforidis; S Khanna et al., *J. Cereb Blood Flow Metab.*, **2011**, 31(11), 2218-2230.
- [27] F Mangialasche; E Westman; M Kivipelto et al., *J. internal medicine*, **2013**, 273(6), 602–621.
- [28] W Yu; M Simmons-Menchaca; A Gapor et al., *Nutr. cancer*, **1999**, 33(1), 26–32.
- [29] WN Yap; PN Chang; HY Han et al., *Br. J. Cancer*, **2008**, 99(11), 1832–1841.

- 
- [30] W Weng-Yew; K Selvaduray; C Ming; K Nesaretnam, *Nutr. Cancer*, **2009**, 61(3), 367–373.
- [31] T Miyazawa; A Shibata et al., *J. Nutr. Biochem.*, **2009**, 20(2), 79-86.
- [32] PN Chang; WN Yap; DT Lee et al., *Nutr. cancer*, **2009**, 61(3), 357–366.
- [33] HK Liu; Q Wang; Y Li et al., *J. Nutr. Biochem.*, **2010**, 21(3), 206-213.
- [34] SU Luk; WN Yap; YT Chiu et al., *International journal of cancer*, **2011**, 128(9), 2182–2191.
- [35] F Aguilar; H Autrup et al., *The EFSA Journal*, **2008**, 640, 1-34.
- [36] D Dutta; UR Chaudhuri; R Chakraborty, *African Journal of Biotechnology*, **2005**, 4 (13), 1510-1520.
- [37] S Mandal; S Yadav; S Yadav; RK Nema, *Journal of Chemical and Pharmaceutical Research*, **2009**, 1(1), 102-104.
- [38] G Vijayalakshmi; M Adinarayana; P Jayaprakash Rao, *Journal of Chemical and Pharmaceutical Research*, **2012**, 4(7), 3574-3582.