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## Determination of Marker Compounds in Combined Herbal Extract-Based Anti-Prostatitis Herbal Product.

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### ABSTRACT

Herbal product (Prostanorm®) is a multi-component herbal drug containing extract of a mixture of St. John's wort (*Hypericum perforatum* L.), golden rod (*Solidago Canadensis* L.), licorice root (*Glycyrrhiza glabra* L.), *Echinacea purpurea* (L.) Moench rhizome and roots in equal quantities. The drug is approved in Russia for treatment of chronic prostatitis in men. Reliable standardization of the product requires quantitative analysis of the main components of the herbal extract. We have previously developed a method of solid phase extraction combined with HPLC for identification and quantitative analysis of marker substances of each herbal component of product, and showed a possibility of their use as markers for control of components' content in the product. Here we describe the applicability of this approach for analysis of the total Prostanorm product. The method includes solid phase extraction on Discovery DSC-18 LT SPE cartridge and subsequent reverse phase HPLC. Drug aliquots were applied onto SPE cartridge, and highly ionic substances (caftaric acid, chlorogenic acid, caffeic acid, chicoric acid, 2-hydroxy-cinnamic acid, rutin, hyperoside) were eluted by 0.2% orthophosphoric acid (fraction 1), while less ionic substances (quercetin, Kempferol and glycyrrhizic acid) were subsequently eluted by ethanol (fraction 2). Both fractions were subjected to two-stage gradient reverse phase HPLC. Optimal chromatographic conditions for separation of marker compounds were established. Method specificity, reproducibility and repeatability were confirmed. The following levels of marker substances (in mg/ml) were found in Prostanorm using the developed assay method: caffeic acid 0.21, caftaric acid 0.83, chlorogenic acid 1.34, chicoric acid 3.25, glycyrrhizic acid 14.98, 2-hydroxy-cinnamic acid 0.47, hyperoside 1.94, kempferol 0.08, quercetin 1.18 and rutin 2.47. SPE/HPLC method was shown to be appropriate for the quality control of the Prostanorm liquid extract.

**Keywords:** herbal product (Prostanorm), marker compounds, solid phase extraction, HPLC, quantitative analysis

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## INTRODUCTION

Herbal product (Prostanorm) is multi-component herbal therapeutic product, which has been developed for treatment of chronic prostatitis in men. Prostanorm is an aqueous-alcoholic (50% ethanol) extract of a mixture of the following herbal raw material: St. John's wort (*Hypericum perforatum* L.), golden rod (*Solidago Canadensis* L.) grass, licorice (*Glycyrrhiza glabra* L.) root, *Echinacea purpurea* (L.) and Moench rhizome and roots taken in equal quantities. The product contains active herbal substances with prostate-tropic activity, which improve prostate microcirculation, urine flow, and show anti-inflammatory, capillary protective, analgesic and antimicrobial effects [1].

Licorice root extract demonstrates significant gonadotropic, androgenic and diuretic activity, and is recommended for prostatitis prevention and treatment [2, 3]. Licorice triterpenoids possess anti-allergic, detoxifying, radical-scavenging and cholesterol-lowering properties [4], and flavonoids show additional antioxidant and antimicrobial activity [5, 6]. Substances extractable from St. John's wort grass in combination with licorice triterpenoids enhance Prostanorm neurotropic activity [7,8]. Flavonoids (rutin, hyperoside) show spasmolytic effect on intestinal smooth muscles, enhance bile outflow and restore normal intestinal peristalsis. Besides, they strengthen capillary walls, thus improving microcirculation in visceral organs [9]. Due to tanning substances, St. John's wort acts as astringent, anti-inflammatory, wound-healing and antimicrobial agent [10]. Moreover, St. John's wort grass contains hypericin and pseudohypericin, which are potent antiviral substances [11]. Golden rod grass extract is used as a component of several herbal products used in cases of prostatitis, prostate adenoma, nocturnal enuresis, cystitis. Golden rod diuretic, anti-inflammatory and "stone-dissolving" effects are ascribed to the grass flavonoids [12]. Extracts of *Echinacea purpurea* roots and rhizomes show immunomodulatory, androgenic and sex potency-improving activity [13].

Previously, we have developed HPLC methods of separation and identification of components of multicomponent herbal products and of each herbal raw material individually [14]. 23 main biologically active substances were identified in individual extracts of the herbal components of the product.

This study was aimed at optimization of chromatographic assay of biologically active marker components and determination of marker substance content in the total extract.

## MATERIALS AND METHODS

Objects: Prostanorm liquid extract 100 mL (batch 040814) was received from Pharmaceutical Industrial Company PharmVILAR (Moscow, Russia)

Reference standards: chlorogenic acid, caftaric acid, glycyrrhizinic acid ammonium salt (glycyram), rutin, caffeic acid, chicoric (also known as cichoric) acid, salicylic acid, 2-hydroxycinnamic acid were purchased from Sigma-Aldrich Chemi GmbH (Steinheim, Germany); hyperoside, were purchased from Merck (Darmstadt, Germany); quercetin, – from Acros Organics (Geel, Belgium), kempferol – from Fluka Chemie AG (Switzerland)

Reagents and solvents: potassium dihydrophosphate, analytical grade was purchased from Avogadro (Moscow, Russia), ortho-phosphorous acid, extra purity grade – from Reachim (Moscow, Russia), acetonitrile, HPLC grade – from Lab-Scan (Gliwice, Poland), dimethylformamide, analytical grade – from ECOS-1 (Russia), absolute ethanol, analytical grade – from Merck (Darmstadt, Germany).

Equipment and chromatographic condition: Chromatography was performed on liquid gradient chromatographic system ProStar (Varian, USA) containing pump ProStar 230 and detector ProStar 325. Columns: Phenomenex Luna C18(2), 250 x 4.6 mm, 5 $\mu$ ; Dr. Maisch Luna 100 C18(2), 250 x 4.6 mm, 5 $\mu$ . Injection volume 20  $\mu$ l (Reodayne injector). Mobile phase flow rate 1.0 mL/min; room temperature. Detection at wavelengths 255 and 320 nm. Mobile phase aqueous component: 0.02M phosphate buffer solution, pH 3, 15 with 0,15% dimethylformamide: The following parameters were calculated for chromatographic system optimization: capacity index ( $k'$ ), efficacy – theoretical plate number (N), selectivity ( $\alpha$ ) and separation ratio ( $R_s$ ).

Solid phase extraction (SPE) system with vacuum chamber (manifold). SPE cartridges –Discovery DSC-18 LT SPE (500 mg), - Supelco, PA USA.

## EXPERIMENTAL

Standard solution and quantification: Standard samples were prepared using reference standards; salicylic acid was used as internal standard. Preparation of standards: solution of each reference standard or salicylic acid 0.1 g/L in 50% ethanol was prepared by sequential 10-fold dilutions of master solution 10 g/L in 50% ethanol, and 20  $\mu$ l of final solutions was injected into chromatographic system.

Preparation of Prostanorm solution: 0.1 mL of Prostanorm extract was diluted by 4.5 ml of 50% ethanol, and 0.4 mL of internal standard solution was added.

Component content was calculated using reference standards according to the following formula:  $X = m_0 \cdot S_x / V \cdot S_0$ ; where X — component concentration in mg/ml,  $m_0$  — weight of reference standard in mg, V — dilution volume,  $S_x$  и  $S_0$  - peak areas of the studied component and reference standard respectively.

Statistical data processing was performed according to [15].

Sample preparation using solid-phase extraction: Cartridges were pre-conditioned by 5 ml of water/ethanol (1:1 v/v) mixture. 0.2 mg of original extract or 0.2 ml of reference standard solutions was loaded onto the cartridge and eluted by 2 eluting solutions: solution 1 – 3 ml of 0.2% aqueous  $H_3PO_4$  solution; solution 2 – 3 ml of 96% ethanol. Each sample obtained by cartridge elution was spiked by 0.5 mL of salicylic acid solution (internal standard). Recovery of extract components (SPE efficacy in %) was calculated from the ratio of peak areas for a component before and after extraction according to the following formula:  $R = (S_x/S_0)100$  (%), where R – component extraction efficacy,  $S_x$  и  $S_0$  – peak areas before and after SPE.

## RESULTS AND DISCUSSION

Prostanorm extract chromatograms registered simultaneously at 255 nm and 320 nm contain 92 peaks with relative area exceeding 0.3% [14]. 23 main components were identified using reference standards. To match the identified components with a certain type of herbal raw material, we previously [14] also performed chromatography of extracts of different types of herbal raw material comprising the product in the same chromatographic conditions. Chromatographic analysis of extracts allowed identification of marker compounds, which serve as witnesses of the given type of herbal raw material in the product extract. Many major components are seen in several types of raw material. For example, chlorogenic acid can be found in St. John's wort grass and Echinacea roots and rhizomes extracts, and both rutin and quercetin flavonoids can be found in St. John's wort grass and golden rod grass extracts. At the same time, a number of compounds are specific for a certain type of herbal raw material: caftaric, chicoric, 2-hydroxycinnamic, caffeic acids are typical for Echinacea, hyperoside - for St. John's wort grass, naringenin and kempferol – for golden rod grass, and glycyrrhizinic acid – for licorice roots. Above listed compounds were also found by authors [16-21] as the main components of extracts of the relevant herbal raw material. These compounds were chosen as markers to characterize each type of herbal raw materials used in Prostanorm formulation.

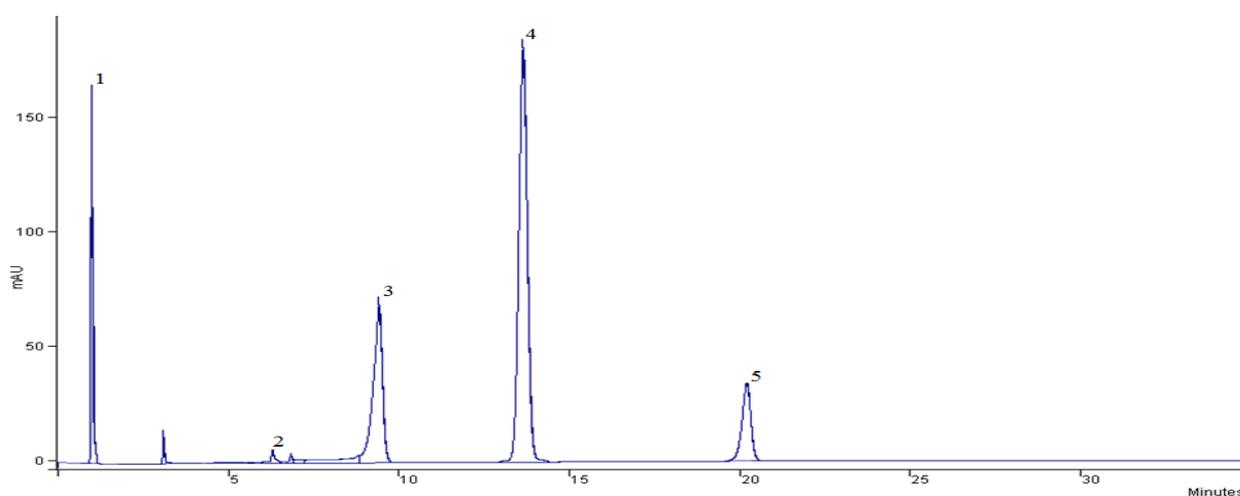
Quantitative analysis of the markers is a necessary and sufficient step for standardization of multi-component products like Prostanorm.

The method of Prostanorm extract analysis [14] is aimed at analysis of the whole range of compounds (~100 substances). With such large number of compounds, capacity and selectivity of resolution of many substance pairs can be insufficient for quantitative analysis. To solve this problem, we utilized a combined approach including sample preparation by solid-phase extraction (SPE) and analysis by HPLC.

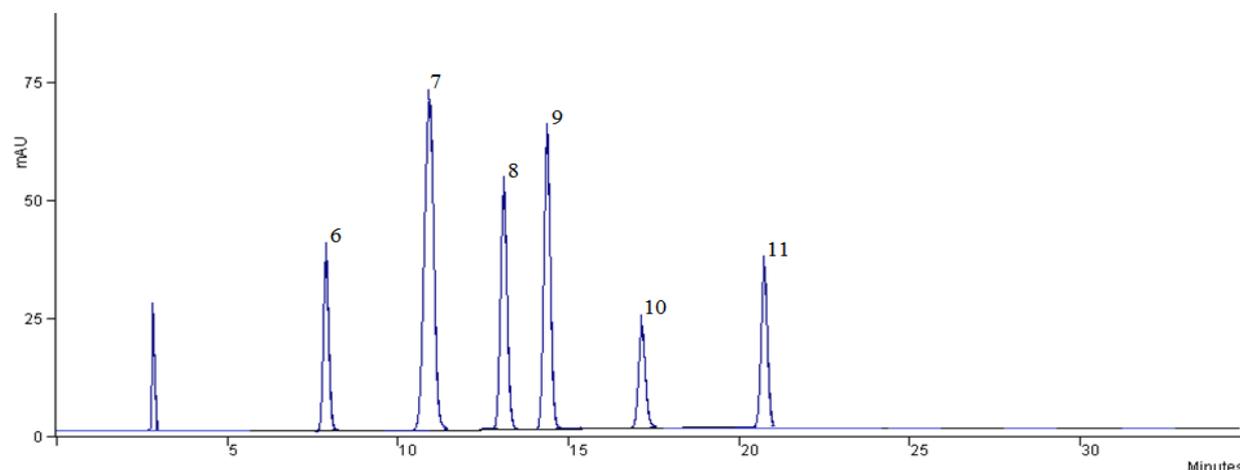
Sample preparation is aimed at marker isolation and removal of accompanying extract components, which interfere with marker analysis. Satisfactory results were obtained by using octadecylsilane sorbent Discovery DSC-18 LT SPE. SPE optimal conditions and efficacy were determined by using a model mixture of the following reference standards: caftaric, chlorogenic, caffeic, chicoric, 2-hydroxycinnamic, glycyrrhizinic acids, hyperoside, quercetine, kempferol, rutin. 0.2 ml sample was completely absorbed on 500-mg cartridge.

Two extracting solvents – 0.2% orthophosphoric acid, pH 2.8 and 95% ethanol were sufficient for fractionation. The more ionic markers (caftaric acid, chlorogenic acid, caffeic acid, chicoric acid, 2- hydroxycinnamic acid, hyperoside, rutin) were eluted by 0.2% $H_3PO_4$  (fraction 1), and less ionic markers (glycyrrhizinic acid, quercetine, kempferol) - by ethanol (fraction 2). SPE efficacy (% recovery) for markers ranged from 69.7% (chlorogenic acid) to 87.3% (glycyrrhizinic acid).

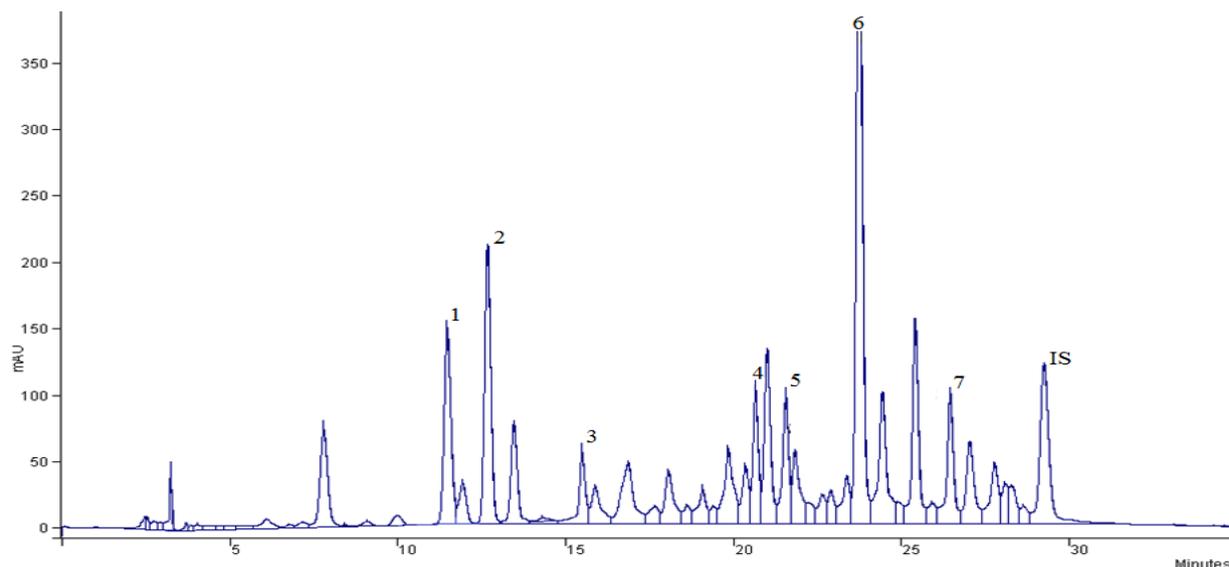
A method involving 2 chromatography runs using the same mobile phase with different gradient parameters was developed for optimization of marker HPLC analysis. To analyze the 1<sup>st</sup> SPE fraction, we used a gradient with initial slow acetonitrile content growth (for optimal resolution of ionic substances) and subsequent abrupt acetonitrile content growth (to remove accompanying substances from the column) (mobile phase: acetonitrile (A) and phosphate buffer, pH 3 with 0.1% dimethylformamide (B); gradient 1 (A:B, v/v): 0 min – 10:90, 5 min 10:90, 25 min – 30:70, 35 min - 90:10). To analyze the 2<sup>nd</sup> SPE fraction containing less ionic substances, we used a gradient with initial abrupt acetonitrile content growth and subsequent mildly sloping increase (mobile phase: acetonitrile (A) and phosphate buffer, pH 3 with 0.1% dimethylformamide (B); gradient 2 (A:B, v/v): 0 min – 35:65, 10 min 35:65, 30 min – 60:40, 35 min - 95:5). Chromatograms of a model marker mixture are shown on fig. 1 and fig. 2, and chromatograms of Prostanorm liquid extract after SPE fractionation are shown on fig. 3 and fig. 4. Fractionation allows for significant “decimation” of chromatogram peaks, thus improving conditions for qualitative analysis.



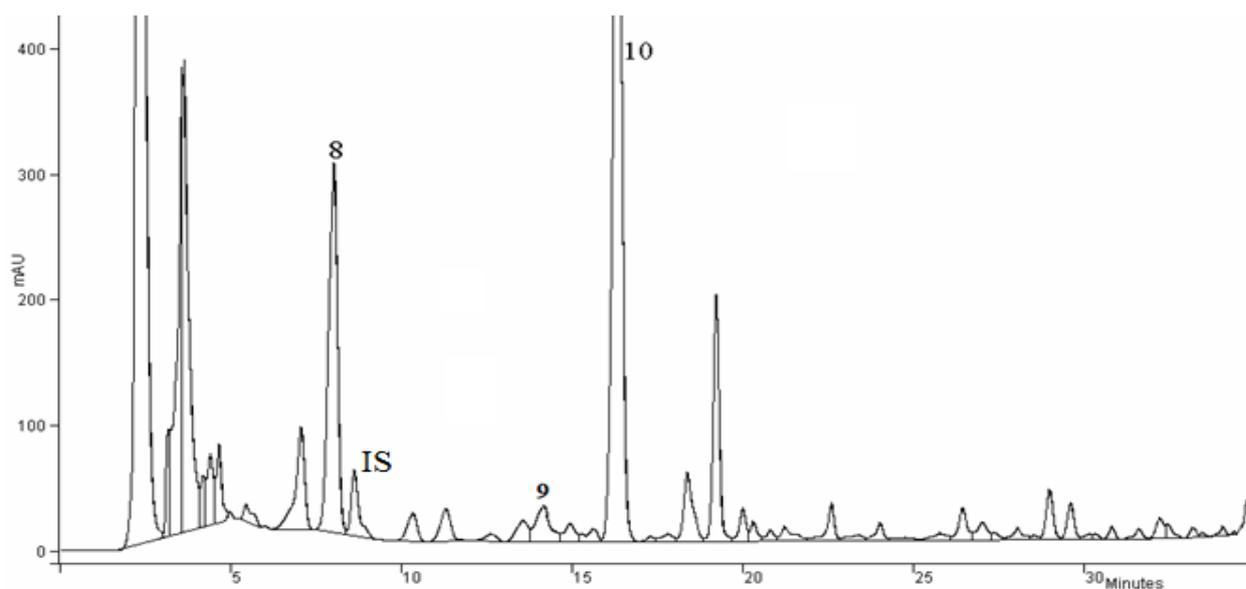
**Fig 1: Chromatogram of reference standard mixture, fraction 1. Detection wavelength 320 nm. Peaks: 1 – ascorbic acid, 2 – caftaric acid, 3 – chlorogenic acid, 4 – caffeic acid, 5 – rutin.**



**Fig 2: Chromatogram of reference standard mixture, fraction 2. Detection wavelength 320 nm. Peaks: 6 – hyperoside, 7 – chicoric acid, 8 – 2-hydroxycinnamic acid, 9 – quercetin, 10 – kempferol, 11 – glycyrrhizinic acid.**



**Fig 3: Chromatogram of Prostanorm liquid extract after SPE sample preparation. Detection wavelength 320 nm. Chromatographic conditions – gradient 1. Peak numbers correspond to substance numbers in table 1. IS – internal standard – salicylic acid**



**Fig 4: Chromatogram of Prostanorm liquid extract after SPE sample preparation. Detection wavelength 255 nm. Chromatographic conditions – gradient 2. Peak numbers correspond to substance numbers in table 1. IS – internal standard – salicylic acid**

Marker analysis by combined SPE and HPLC has been validated for specificity, repeatability and reproducibility. These parameters of system suitability were assessed both for analysis of model marker mixture, and for analysis of intact Prostanorm extract. Capacity index  $k'$  fell within optimal range 3.6-9.6, efficacy  $N$  was not less than 20,000 theoretical plates for each peak, and selectivity  $\alpha$  and resolution index  $R_s$  values calculated for adjacent peaks were optimal for the model mixture (table 1). As for intact Prostanorm extract, addition low-height peaks could be seen due to complex nature of the matrix, and they slightly impaired the separation quality, though it remained sufficient for qualitative analysis.

**Table 1: Parameters of chromatographic separation of marker compounds after solid-phase extraction, and their content in Prostanorm (PPC PharmVILAR, batch # 040814)**

No	Substance	Efficacy, N theor. plates (n=5)	Capacity index k' (n=5)	Selectivity $\alpha$ (n=5) <sup>a</sup>	Resolution factor R <sub>s</sub> (n=5) <sup>a</sup>	Relative error of peak square value,% (P = 0.95, n=9)	Content X <sub>m</sub> ± Δx <sub>m</sub> , mg/ml (P = 0.95, n=9)
1	Caftaric acid	20850	3,6	1,09	5,7	5,8	0,83±0,05
2	Chlorogenic acid	34100	4,2	1,09	5,1	3,1	1,34±0,04
3	Caffeic acid	59200	5,2	1,04	2,7	5,9	0,21±0,01
4	Rutin	111600	7,1	1,03	1,2	7,5	2,47±0,16
5	Hyperoside	107000	7,6	1,03	1,2	3,2	1,94±0,06
6	Chicory acid	98100	8,3	1,03	4,1	2,3	3,25±0,07
7	2-hydroxy-cinnamic acid	119600	9,6	1,03	3,5	5,7	0,47±0,03
8	Quercetin	38340	2,3	1,03	2,8	1,1	1,18±0,02
9	Kempferol	57910	4,6	1,05	4,4	6,8	0,08±0,01
10	Glycyrrhizinic acid	28310	5,6	1,03	6,6	4,1	14,98±0,64

a-Towards an adjacent peak nearest to the studied one; substances 1-7 – gradient 1; substances 8-10 – gradient 2

Reproducibility of peak areas as measured by standard error of the mean value (relative standard deviation) was calculated for different substances in the model mixture of reference standards and fell within ±2% range (for example: chlorogenic acid 1.6%, rutin 1.8%, quercetin 1.1%, glycyrrhizinic acid 0.4%).

Method specificity describes the possibility to reliably determine a marker's content in the presence of other extract components. The model mixture of reference standards was subjected to SPE in the same conditions as intact Prostanorm extract. Marker peaks were well separated from peaks of accompanying substances, and signal/noise ratio exceeded 100 for all compounds.

Method repeatability was assessed by the degree of convergence of the results of multiple (not less than 9) repeated measurements. Data on reproducibility of marker peak areas in Prostanorm chromatogram are presented in table 1 for example, one of the production batches (PPC PharmVILAR, batch # 040814). These data allow a conclusion about good reproducibility of the method. Relative error of the mean peak area for some substances was higher when analyzed in intact Prostanorm extract as compared to model mixture (the largest difference was seen for rutin – 7.5% in intact Prostanorm extract and 1.8% in model mixture), but it remained acceptable for quantitative analysis. The quantitative content of marker components in the batch is also shown in table 1.

## CONCLUSION

The method provides estimation of the identified marker compounds of each type of herbal raw material present in the extract of multi-component product Prostanorm, has been developed. A combined technique included procedure of Solid Phase Extraction and HPLC method for quantitative analysis of Prostanorm indicator substances. The procedure of SPE have been shown to receiving the best extractions results of markers from extract of drug Prostanorm. The optimal conditions of chromatographic separation of indicator compounds have been determined. The method was shown to be appropriate, in terms of reproducibility, specificity, repeatability and can be used for authentication and quality control of extracts. Combined SPE/HPLC method of marker assay was tested by analysis of samples of production batches. Prostanorm marker components are (in descending order) glycyrrhizinic acid, chicoric acid, rutin, hyperoside, chlorogenic acid, quercetin, caftaric acid, 2-hydroxy-cinnamic acid, caffeic acid, kempferol.

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