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HPLC-Analysis of narcissin in flowers of *Calendula officinalis*

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Abstract
With the using of HPLC the method of the quantitative determination of the dominant flavonoid, narcissin (3-O-rutinoside of isorhamnetin) in the flowers of *Calendula officinalis* L. there was developed. It was determined that the content of narcissin in the in the flowers of this plant varies from 0,68±0,02% to 0,97±0,03%. The developed method of HPLC-analysis can also be used to determine the authenticity of the flowers of *Calendula officinalis* by means of the detection of narcissin, which has the diagnostic significance.

Keywords: marigold, *Calendula officinalis* L., flowers, flavonoids, narcissin, HPLC, standardization.

1. Introduction
The flowers of the marigold (*Calendula officinalis* L.) are widely used in the domestic and foreign medicine as anti-inflammatory, regenerating, choleretic, expectorants phytopharmaceuticals [1-3]. The known pharmacological properties of the remedies from the flowers of *Calendula officinalis* cause the flavonoids, carotenoids and saponins [2, 3]. However, despite a good degree of the knowledge of the chemical composition of the raw material of this plant, is still not fully solved the problems of standardization, particularly in terms of quantitative estimation of biologically active compounds. So, in the pharmacopoeial monograph on the flowers of *Calendula officinalis*, are included in the State Pharmacopoeia of the USSR XI edition, provides the definition only of the content of extractives [1]. In accordance with the European Pharmacopoeia the quantitative determination of the content of total flavonoids is carried out by spectrophotometry at a wavelength of 425 nm, calculated as hyperoside [4], however, this flavonoid is not contained in the flowers of *Calendula officinalis*. The standardization of the marigold flowers in the State Pharmacopoeia of the Republic of Kazakhstan and of Ukraine [5, 6] is formulated by analogy with the approaches of the European Pharmacopoeia. The quality of preparations "Calendulae tincture" and "Calendulae liquid extract" [2] is estimated based on the total content of oxidized substances that, in our opinion, does not allow to objectively carry out their standardization. Yet more inconsistency in approaches to standardization is detected when determining the authenticity of flowers and preparations of *Calendula officinalis*, which is carried out, usually by the detection of rutin, caffeic acid and chlorogenic acid [4-6], which are non-diagnostic compounds for the raw material of this plant.
Previously we have found that the dominant and diagnostic compound *Calendula officinalis* flowers is a flavonoid narcissin (3-rutinoside of isorhamnetin) [7-9], making it an appropriate definition of this component not only TLC, but by HPLC.
There are reports on the application of the HPLC method of analysis for standardization of raw materials of *Calendula officinalis* [3, 10]. According to some authors, the analysis of the extraction of the flowers of *Calendula officinalis* by HPLC is needed by the identification of quercetin with the definition of the height of the peak of this compound, as well as using standard sample of quercetin [3].
In our opinion, quercetin is not diagnostic compound for flowers of *Calendula officinalis*, because this component contains in trace amounts. In addition, this flavonoid is the most common flavonoid aglycone in plants. Other researchers for the purposes of the HPLC analysis offer use a marker such components as typhaneoside [isorhamnetin-3-O-α-L-rhamnopyranose-(1→2)-α-L-rhamnopyranose-(1→6)-β-D-glucopyranoside], narcissin, isorhamnetin-3-O-glucoside and isorhamnetin-3-O-(6[11]-acetyl)-glucoside [10], and provides for

the determination of total flavonoids that from the point of view pharmacopoeial analysis is problematic.

In our view, the dominant and diagnostic significant flavonoid is narcissin, and the definition of it is appropriate for the purposes of the standardization of raw materials and preparations of *Calendula officinalis*.

The aim of our research is to develop the methods of the quantitative determination of narcissin in the flowers of *Calendula officinalis* using high-performance liquid chromatography.

2. Materials and methods

The objects of study were the flowers of *Calendula officinalis* harvested in August 2013 and 2014 on the Botanical garden of Samara State University (sort "Kalta"), as well as commercial samples of raw materials (OAO "Krasnogorskleksredstva", ZAO "Samaralektravy"). Air-dried plant material was subjected to extraction with 70% ethanol in the ratio of raw materials – extractant is 1:30 in a boiling water bath for 60 minutes.

Chromatographic separation of water-alcohol extracts of *Calendula officinalis* flowers was carried out using a liquid chromatograph "Biotronic" with UV detector in gradient mode. As reference substances, standard samples were used narcissin (3-O-rutinoside of isorhamnetin), rutin (3-O-rutinoside of quercetin), quercetin and isoquercitrin (3-O-β-D-glucopyranoside of quercetin).

3. Results and Discussion

The results of HPLC analysis indicate that in the studied conditions chromatography of narcissin with the magnitude of retention time about 37,42 minutes (peak 1) well separated from other components of the flowers of *Calendula officinalis* (Fig. 1), which allows this method can be recommended for identification of raw materials of the marigold, and for purposes of standardization of preparations on the basis of the raw material of this plant. During the chromatography of the working standard sample of narcissin by HPLC the retention time of the analyte amounted 37,83±0,10 minutes (Fig. 2), which confirms the correctness of component separation in aqueous-alcoholic extract of flowers of *Calendula officinalis* (Fig. 1).

It is determined that the content of the dominant compounds (narcissin) in the flowers of *Calendula officinalis*, established by the HPLC method, varies from 0,68±0.02% to 0,97±0,03%. In these conditions in the analyzed extract also indicates the presence of a peak of rutin (0,28%±0,03%) (peak 2), with a retention time of 32.64±0,12 minutes, and in minor amounts are found isoquercitrin (retention time is 43,46±0,13 minutes) and quercetin (43,87±0,10 minutes) (peaks 3 and 4 respectively) (Fig. 1).

Thus, in the course of the study was developed the method for the quantitative determination of the dominant flavonoid narcissin by the means of the reversed-phase HPLC. The content of narcissin in the flowers of *Calendula officinalis,* established by the HPLC method, varies from 0,68±0,02% to 0,97±0,03%. The relative degree of the determination of narcissin in developed method with confidence probability 0,95 is no more than ±4,51%.

Given the specificity of narcissin for flowers of *Calendula officinalis*, we consider it reasonable to use of the HPLC method to determine the authenticity of raw materials and preparations of plants for the detection of this flavonoid, which has diagnostic value.

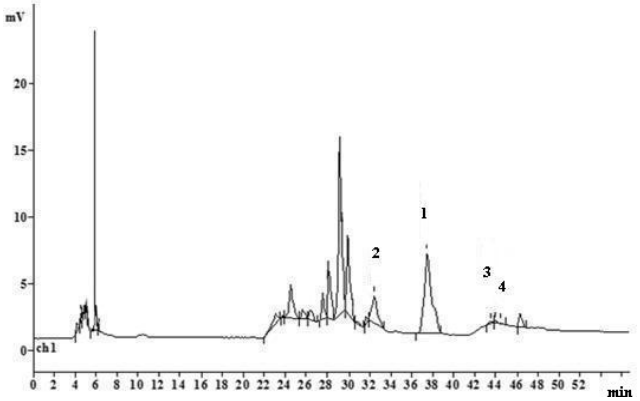

Fig 2: HPLC chromatogram of the water-ethanol extract from *Calendula officinalis* flowers.

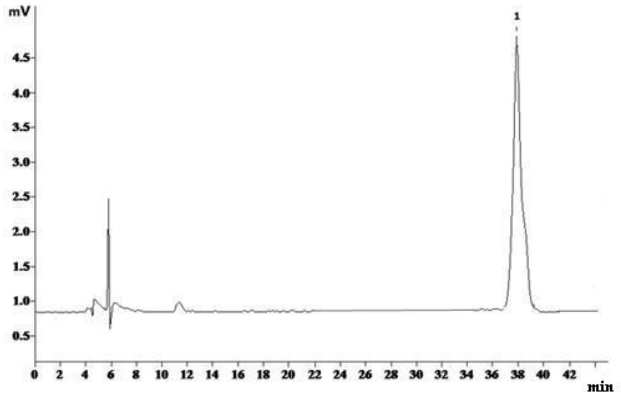

Fig 2: HPLC chromatogram of the alcoholic solution of narcissin (1).

3.1. The method for the quantitative determination of narcissin in the flowers of *Calendula officinalis*. Weigh accurately about 1 g of the pulverized raw material was placed in a flask with a ground joint with a capacity of 50 ml, add 30 ml of 70% ethahol. The flask is closed with a stopper, is weighed with an accuracy of ±0.01 g. The flask is attached to the reverse refrigerator and heated in a boiling water bath for 60 minutes. Then the flask was cooled for 30 minutes, cover the same tube, weighed again and fill the missing extractant to the initial weight. The extract was filtered through a paper filter. 20 µl of the resulting aqueous-alcoholic extract of *Calendula officinalis* flowers is injected into the liquid chromatograph "Biotronic" with UV detector. The elution of the introduced samples are performed in reversed-phase chromatography in column "Phenomenex Luna C18(2)" (250x2,0 mm), in a gradient three-stage mode: eluent system (mobile phase): 0,01 M KH_2PO_4 solution, acidified with H_3PO_4 to pH 3,0 (buffer solution), and methanol, first the mixture of buffer solution - methanol the ratio 90:10, from 10th minutes - 50:50 and from 42nd minutes – 30:70). The flow rate of elution is 0.6 ml/min. UV detection is carried out at a wavelength of 254 nm. In parallel in the liquid chromatograph is injected 20 µl of the standard sample solution B of narcissin and chromatographic under the same conditions. On the chromatogram of the test solution define the peak area of narcissin with a retention time of about 37 minutes and calculate the average peak area along three parallel determinations. Measure the peak area of narcissin on the chromatogram of the solution of the working standard sample of narcissin and calculate the average peak area for three parallel definitions.

The content of narcissin in the flowers of *Calendula officinalis*

in percent (X) in terms of absolutely dry raw material is calculated by the formula:

$$X = \frac{S \times m_i \times V \times 5 \times V_1 \times 100 \times 100}{S_i \times m \times V_o \times 25 \times V_2 \times (100 - W)},$$

Where:
S - the area of the peak of narcissin on the chromatogram of the test solution;
S_o - the peak area of narcissin on the chromatogram of the standard sample;
m – the weight of raw material, g;
m_o - the weight of narcissin in the solution A, g;
V – the volume of extract, ml;
V_1 - the volume of injected of the test solution, µl;
V_o - the volume of the solution of narcissin (solution A), ml;
V_2 - the volume of injected solution B of narcissin, µl;
W – the loss of drying of raw material, %.
Notes: 1. *Solution preparation of working standard sample of narcissin*. About 0.02 g (a precisely weighed) of the standard sample of narcissin placed in a volumetric flask with a capacity of 25 ml, dissolved in 20 ml of 95% ethanol to bring the volume of solution up to the mark with the same solvent and mix (solution A). 5 ml of solution A is placed in a volumetric flask with a capacity of 25 ml, bring the volume of solution 95% ethanol to the mark and mix (solution B). The volume of injected sample is 20 µl.
2. *Preparation of eluent system for HPLC*. A suspension of potassium hydrophosphate (analytical grade, GOST 2493-75) 1,36 g is transferred to a volumetric flask with a capacity of 1 liter, dissolve in water and dilute to the mark with water, mix. The resulting solution is acidified with a solution of phosphoric acid to a pH of 3,00±0,01. The solution was filtered through a membrane filter with a pore diameter of not more than 0,4–0,5 µm. The solution is degassing using ultrasonic bath before measurement. The solution should be used within 1 month.
3. *Check the suitability of the chromatographic system*. Chromatographic system is acceptable if the following conditions are true: the efficiency of a chromatographic column, calculated by peak of narcissin, shall be not less than 3000 theoretical plates.

4. Conclusions

1. The developed new approaches to the standardization of the approach to standardization of the flowers of *Calendula officinalis*, which consists in the determination of narcissin using of HPLC.
2. The content of narcissin in the flowers of *Calendula officinalis,* established by the HPLC method, varies from 0,68±0,02% to 0,97±0,03%.
3. Given the specificity of narcissin for flowers of *Calendula officinalis*, we consider it reasonable to use of the HPLC method to determine the authenticity not only of raw materials, but also of the preparations of investigated plants for the detection of this flavonoid, which has diagnostic value.

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