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DEVELOPMENT AND VALIDATION OF METHODS FOR THE QUANTITATIVE DETERMINATION OF ARBUTIN IN COWBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES

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The paper presents the results of the development and validation of the method of analysis of arbutin in cowberry leaves by capillary electrophoresis. Sample preparation of the extraction was carried out in accordance with the pharmacopoeial monograph FS.2.5.0063.18 of SP XIV edition. It was found that the UV-spectrophotometry method proposed in the pharmacopoeial monograph determines the quantitative content of not only the arbutin component, but the total amount of phenol-glycosides. The standard content of the arbutin component in the leaves of cowberries by capillary electrophoresis was determined as at least 4%. At the same time, the results of the quantitative content of arbutin are confirmed by using the HPLC method. The method of quantitative determination of arbutin in cowberry leaves by capillary electrophoresis was validated according to the "linearity", "precision" and "intra-laboratory precision", "correctness" parameters. The developed method can be recommended for the analysis of cowberry raw materials.

Keywords: cowberry (*Vaccinium vitis-idaea* L.) leaves, arbutin, capillary electrophoresis, HPLC, UV-spectrophotometry

Currently, there is a tendency to increase the use of chromatographic methods for the analysis of medicinal plant raw materials. In foreign countries, the main method is high-performance liquid chromatography (HPLC), which serves as the basic method of quality control of medicinal plant raw materials. In the pharmacopoeia analysis on the territory of the Russian Federation, 107 individual monographs on medicinal plant raw materials are used, and 7 of which use the HPLC

method to assess the authenticity of raw materials and provide the quantitative determination, a large number of methods are based on electron spectroscopy. This is due to the use of expensive equipment, columns and high-purity solvents (acetonitrile, methanol, etc.) in the HPLC analysis. The spectrophotometric method of analysis is used more often due to the availability of equipment and reagents, but it is worth considering that when choosing this method, we judge the amount of substances in terms of the dominant component. The quantitative content of the major component, in contrast to the HPLC method, cannot be determined by spectrophotometry [1,2].

Capillary electrophoresis (CE) is one of these separation analysis methods that can be used to determine the component composition and the quantitative content of each component. Through the CE, a high efficiency of separation of substances is achieved while the use of expensive reagents and chromatographic columns is not required (the analysis is carried out in a quartz capillary). Thus, it is worth noting the advantages of using CE over HPLC – this is a significantly lower price for a single analysis, the presence of more stable analysis conditions, and the rapidity of the analysis. However, to date, this method has not found the use for the analysis of medicinal plant raw materials.

We have developed a method for the quantitative determination of arbutin by capillary electrophoresis. Previously, the use of capillary electrophoresis for the quantitative determination of arbutin in water extraction from the leaves of cowberry and bearberry was described in the literature [3]. However, we focused on the sample preparation of the extraction of cowberry leaves according to the pharmacopoeial monograph in SP of XIV edition, since the largest amount of arbutin is extracted by 70% ethyl alcohol.

Cowberry (*Vaccinium vitis-idaea* L.) leaves in the SP of XI edition were standardized for the content of arbutin by the method of iodometric titration, in the SP of XIV edition the method of electron spectroscopy is used after preliminary purification of water-alcohol extract using a column with aluminum oxide. The lower limit is standardized as at least 4.5% of arbutin in terms of air-dry raw materials [4].

The purpose of this work is development and validation of methods for quantitative determination of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves by capillary electrophoresis.

MATERIALS AND METHODS

The objects of analysis are cowberry (*Vaccinium vitis-idaea* L.) leaves harvested in various regions of the Russian Federation: Kabardino-Balkar Republic (KBR), Zolsky District, Harbas River bank, autumn 2019; Perm Krai, Kudymkarsky District, spring 2018; Bryansk Region, spring 2018; Irkutsk Region, Bratsky District, Novoe Prirechye village, summer 2019; Altai Krai, Pavlovsky District, autumn 2019; Moscow Region, Mytishchi District, spring 2019. The harvest was carried out in accordance with the requirements of the FS.2.5.0063.18 of SP XIV edition

Sample preparation for determining the content of arbutin in the analyzed objects was carried out in accordance with the requirements of the FS.2.5.0063.18 of SP XIV edition, section "Quantitative determination" [4].

Initially, the electronic spectra of extracts from cowberry (*Vaccinium vitis-idaea* L.) leaves were measured using a SF-2000 spectrophotometer (OKB Spektr JSC, Russia) in the wavelength range from 200 to 400 nm. The calculation was carried out by the value of the specific light absorbance of arbutin.

Further, the content of arbutin was determined by the CE method. To do this, we took the previously obtained extract (FS.2.5.0063.18) that was purified using a column with aluminum oxide and without pre-cleaning. The analysis was

carried out using a Kapel-105M device (Lumex-marketing JSC, Russia) with a quartz capillary (capillary diameter 75 microns, L_c/L_{ef} = 50/60 cm). The quartz capillary was pre-washed sequentially with purified water, 1 M of sodium hydroxide solution, purified water, 1 M of hydrochloric acid solution, purified water and buffer solution. Buffer and washing solutions were filtered through a Vladipor membrane filter of MFAS-B-4 type (STC "Vladipor", Russia), disc diameter 25 mm. The analyzed extract and buffer solution were centrifuged at 8000 rpm, for 5 min. Analysis conditions: sample introduction was carried out hydrodynamically at 150 mbar · s; detection at a wavelength of 254 nm; voltage: +20 kV; capillary temperature: 20°C; electrolyte: 10 mM of borate buffer solution with pH=9.8; analysis time: 10 minutes.

The "Elforan" program (version 3.2.5) was used for processing electrophoregrams. The arbutin content in the extract was calculated using the equation of the calibration graph, which was constructed during the analysis of arbutin reference standard (RS) (Sigma-Aldrich) in the analytical range of concentrations from 0.01 to 0.2 mg/l.

Validation of the method of quantitative determination of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves by capillary electrophoresis was carried out in accordance with SP XIV in terms of linearity, precision (repeatability and intra-laboratory precision) and correctness [3].

To confirm the results, a reversed-phase HPLC variant was used. The analysis was performed using a Steyer chromatograph (Aquilon, Russia) equipped with a Luna C18 column (Phenomenex, USA) with dimensions of 150 × 4.6 mm (sorbent grain size of 5 µm) in the isocratic elution mode. The mobile phase was a mixture of 0.05 M of solution of phosphoric acid and acetonitrile in a ratio of 97:3, the flow rate was 1 ml/min, the volume of the injected sample was 20 µl, the peaks were detected spectrophotometrically at 280 nm. Sample preparation included dilution of 1 ml of crude alcohol extracts from cowberry (*Vaccinium vitis-idaea* L.) leaves with a mobile phase of up to 10 ml. The quantitative content of arbutin in medicinal plant raw materials was determined using the arbutin peak area on chromatograms of reference standard solutions (Sigma-Aldrich, 0.003216% of reference standard solutions in 70% ethyl alcohol).

RESULTS AND DISCUSSION

The spectral analysis of the studied objects showed that the maxima of light absorbance of the extracts coincided with the maximum of light absorbance of arbutin (283±2 nm) (Fig. 1).

The results of quantitative determination of cowberry (*Vaccinium vitis-idaea* L.) leaves from various places of natural vegetation are presented in Table 1.

When studying the optimal conditions for the analysis by capillary electrophoresis, we were faced with the question that arbutin can be present in the raw material in the form of conjugates, for example, in the form of 2-O-caffeoyl arbutin, as well as together with methylarbutin and hydroquinone which is its decomposition product [5]. Some of them can make an important contribution to the spectral method of analysis, since, being phenologlycosides, they are not adsorbed on the column with aluminum oxide

In this regard, the pH of the electrolyte was selected in order to optimally separate the peaks of arbutin and related components. It was found that when using pH=9.6 of 0.01 M borate buffer solution, the best separation of the arbutin peak from other phenoglycosides in the extract is achieved (Fig. 2).

In addition, at pH=9.6, the technique allows us to obtain stable results, since in the pH range of ±0.2, the efficiency of the arbutin peak varies slightly (Table 2).

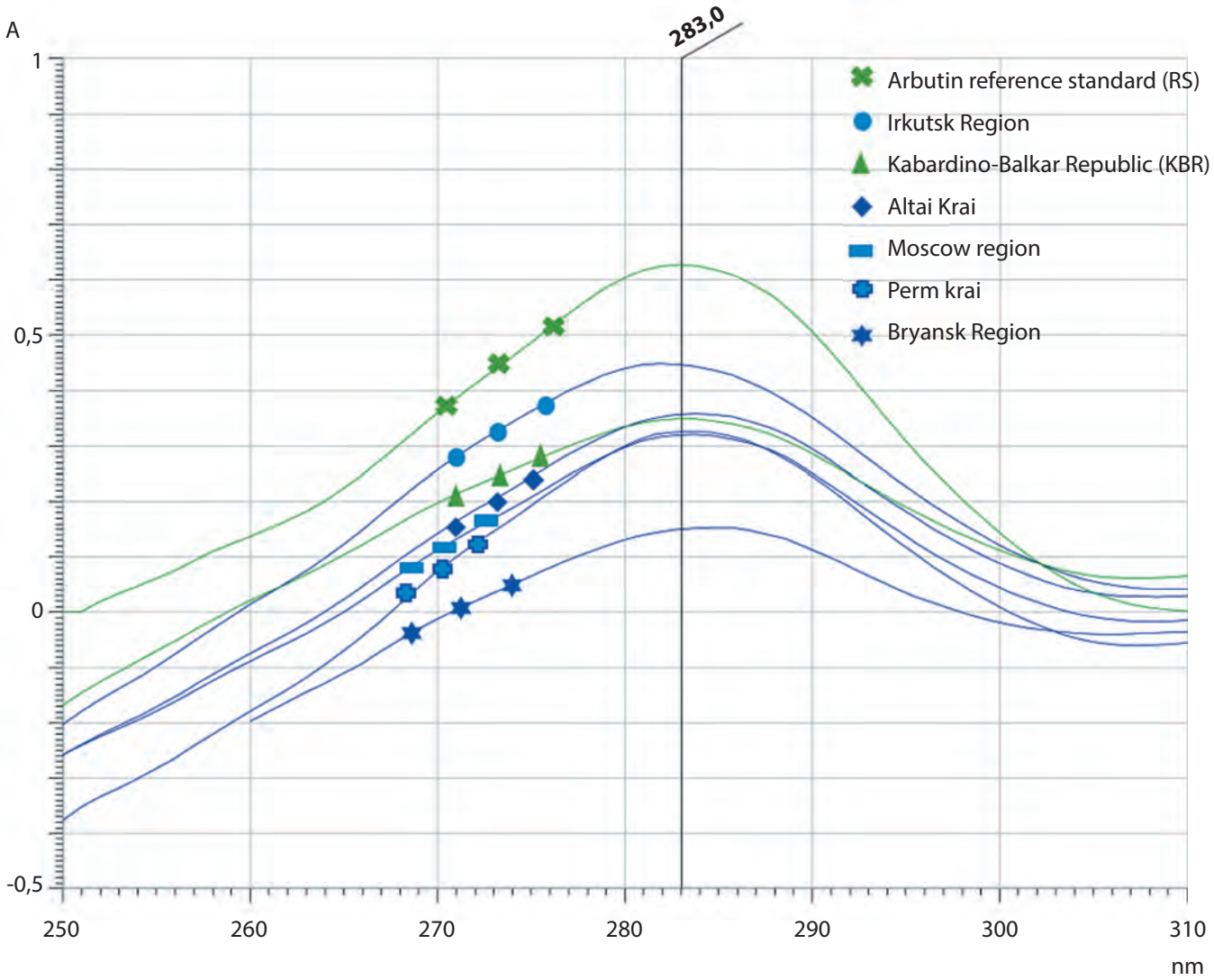

FIG. 1. *Absorption spectra of the solution of arbutin reference standard (RS) and water-alcohol extracts (extraction solvent – 70% ethyl alcohol) from cowberry (Vaccinium vitis-idaea L.) leaves harvested in different places of growth*

Table 1

RESULTS OF QUANTITATIVE DETERMINATION OF ARBUTIN IN COWBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES BY UV-SPECTROPHOTOMETRY

Harvesting Region	n	f	$\bar{x}$	S_x	$S_{\bar{x}}$	$t_{(p, f)}$	Δx	ε, %
KBR (autumn 2019)	7	6	8.50	0.1899	0.0718	2.45	0.18	2.07
Perm Krai (spring 2018)	7	6	7.91	0.2566	0.0970	2.45	0.25	3.11
Bryansk Region (spring 2018)	7	6	5.74	0.2093	0.0791	2.45	0.20	3.50
Irkutsk Region (summer 2019)	7	6	11.67	0.5503	0.2080	2.45	0.51	4.36
Altai Krai (autumn 2019)	7	6	8.31	0.2999	0.1134	2.45	0.28	3.34
Moscow Region (spring 2019)	7	6	7.83	0.1907	0.0721	2.45	0.18	2.26

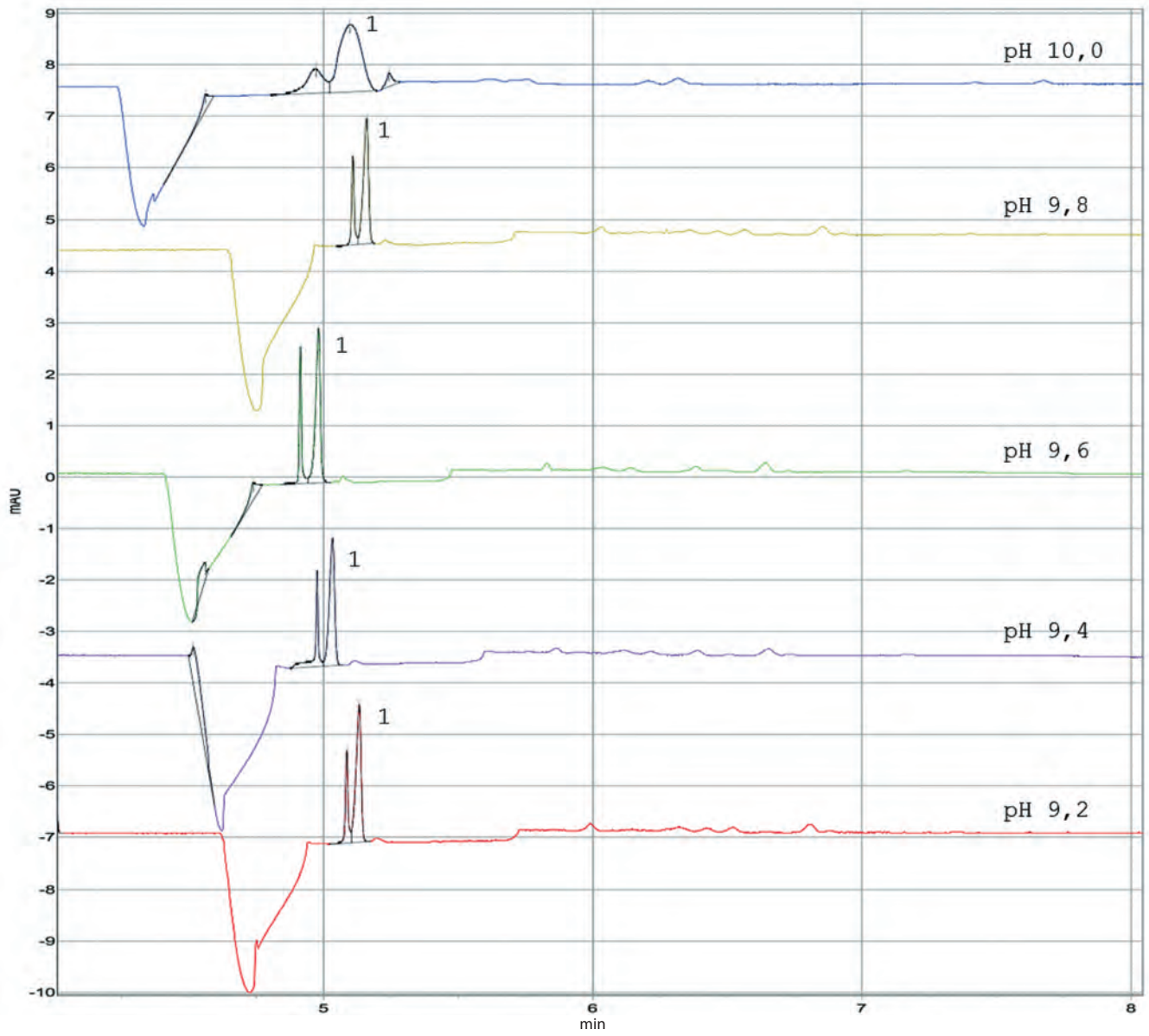

FIG. 2. *Electrophoregrams of water-alcohol extract (extraction solvent – 70% ethyl alcohol) from cowberry (Vaccinium vitis-idaea L.) leaves (Kabardino-Balkar Republic), obtained at different pH values of 0.01 M of borate buffer solution (1 – arbutin)*

Further, according to the method, the extracts obtained by cleaning using a column with aluminum oxide and the crude extracts were analyzed under the selected conditions (Fig. 3)

The difference between the content of arbutin in the crude and purified extract is within the permissible error of the method used, so it is advisable to conduct an analysis by capillary electrophoresis without pre-cleaning the extract from the accompanying phenolic compounds, which will reduce the analysis time.

Table 2

RESULTS OF EVALUATING THE EFFECTIVENESS OF THE ARBUTIN PEAK WHEN USING A BORATE BUFFER SOLUTION WITH DIFFERENT PH VALUES

Effectiveness, thousands of theoretical plates				
pH=9.2	**pH=9.4**	**pH=9.6**	**pH=9.8**	**pH=10.0**
414	418	434	439	18

Thus, we have proposed the following scheme for obtaining and analyzing the extract of cowberry (*Vaccinium vitis-idaea* L.) leaves for the content of arbutin by capillary electrophoresis: 0.5 g of raw materials (exact sample weight) with a particle size of 1 mm is extracted with 100 ml of 70% ethyl alcohol, after weighing a flask with a sample and an extraction solvent with an error of ±0.01 g, in a boiling water bath with a backflow condenser for 45 minutes. After extraction, the extract is cooled, the flask is weighed and, if necessary, brought to the original mass with 70% ethyl alcohol. The extract is filtered through a paper filter moistened with 70% ethyl alcohol, discarding the first 10 ml of the filtrate. Then the 3 ml volume aliquot of the resulting filtrate is introduced into a 25 ml volumetric flask and brought to the mark with 70% ethyl alcohol, mixed. Next, 1 ml of extract is centrifuged at 8000 rpm for 5 minutes

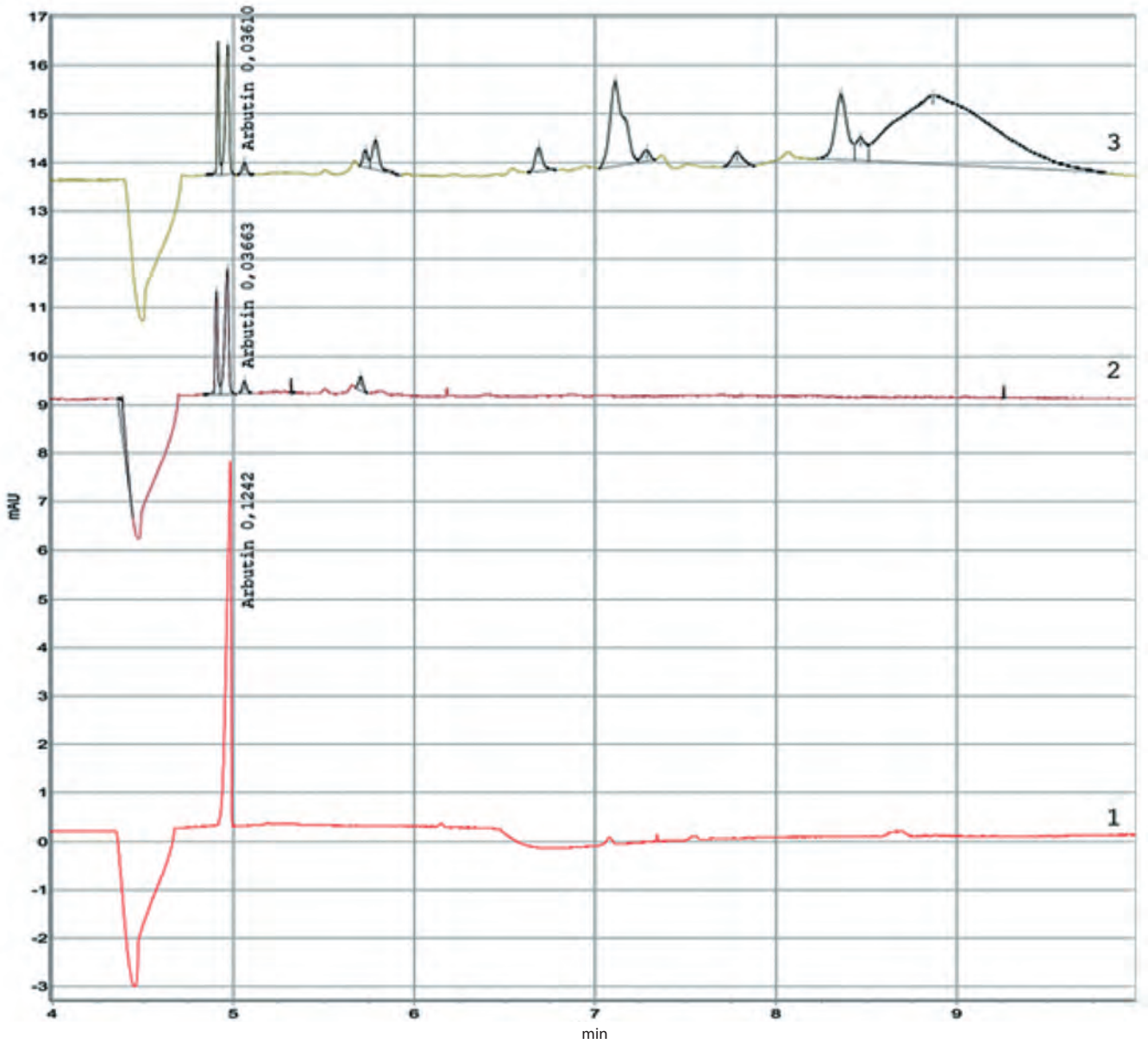

FIG. 3. *Electrophoregrams: 1 – solution of arbutin reference standard (RS); 2-purified water-alcohol extract (extractant – ethyl alcohol 70%) from common lingonberry leaves (Kabardino-Balkar Republic); 3 – crude water-alcohol extraction (extraction solvent – 70% ethyl alcohol) from cowberry (Vaccinium vitis-idaea L.) leaves (Kabardino-Balkar Republic). Arbutin concentration, mg/ml*

and analyzed according to the selected conditions.

The content of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves in terms of air-dry raw materials is calculated by the equation:

$$X, \% = \frac{C_{\frac{mg}{ml}} \times 100 \times 25 \times 100 \times 100}{1000 \times a \times 3 \times (100 - W)} \quad (1),$$

where $C_{\frac{mg}{ml}}$ – the concentration of arbutin in the extract, calculated according to the calibration graph (Fig. 4); a – raw material weight, g; W – raw material moisture, %.

The developed methodology was further subjected to validation evaluation according to the GPM "Validation of analytical methods" [4].

The linearity of the method was determined by the linear nature of the dependence of the analytical signal (peak area) on the concentration of the reference standard of arbutin in solution in the concentration range of 0.083–0.01 mg/ml.

A linear dependence was observed in the analytical range of arbutin concentrations from 0.08 to 0.01 mg/ml, the correlation coefficient was 0.999, which meets the requirements.

The "precision" parameter was evaluated as repeatability (convergence).

The repeatability of the method was evaluated in 7 repetitions conducted by one analyst on one day, using one device and using the same reagents (Table 4).

The relative standard deviation was determined, which should not exceed 10% [6,7]. This value does not exceed 2.7%.

Intra-laboratory (intermediate) precision was determined in one laboratory, but on different days and by two executors for 3 samples of raw materials in 3 repetitions (Table 5).

The relative standard deviation should not exceed 10% [6,7]. In this case, it did not exceed 4.64%. The resulting data show that the method corresponds to the "precision" parameter.

The analytical method is considered as correct if the results of the experiment are within

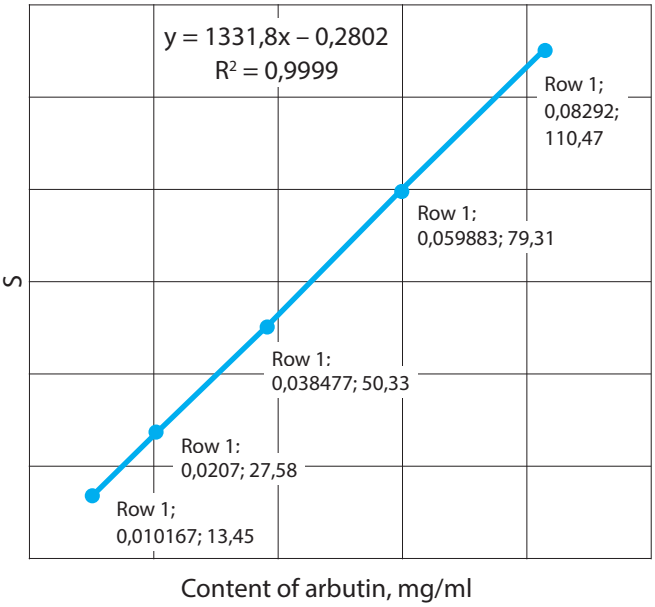

FIG. 4. *Calibration graph of the linear dependence of the peak area of the reference standard of arbutin on its concentration in solution*

the confidence interval of the average result obtained by the method to be validated. The Student coefficient is also calculated and the value of this coefficient should not be greater than the table value for the corresponding sample size ($t_{cal} < t_{tab}$). The correctness was determined by adding a known concentration of a reference standard of arbutin to the test extract obtained from a 0.3 g sample of raw materials with an arbutin content of 60% of the total concentration in the raw material, up to an arbutin content of 80%, 100% and 120%. The ratio of the resulting content

Table 3

THE RESULTS OF THE EVALUATION OF THE "LINEARITY" PARAMETER IN THE VALIDATION OF THE METHOD OF QUANTITATIVE CONTENT OF ARBUTIN IN COWBERRY (VACCINIUM VITIS-IDAEA L.) LEAVES BY CAPILLARY ELECTROPHORESIS

f	$\bar{x}$	$\bar{y}$	b	a	R
4	0.0424	56.23	1331.8	0.2802	0.999

Table 4

RESULTS OF THE EVALUATION OF THE "REPEATABILITY" PARAMETER IN THE VALIDATION OF THE METHOD OF QUANTITATIVE CONTENT OF ARBUTIN IN COWBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES BY CAPILLARY ELECTROPHORESIS

Experiment No.	Sample weight, g	Arbutin peak area, mAU·s	Content of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves, %	Metrological characteristics
1	0.5020	51.64	6.99	$\bar{x} = 6.73\%$ $S = 0.1938$ $S_{\bar{x}} = 0.0733$ $\Delta\bar{x} = 0.18$ $\bar{x} \pm \Delta\bar{x} = 6.73 \pm 0.18$ $\bar{\varepsilon} = \pm 2.67\%$
2	0.5015	49.25	6.67	
3	0.5034	48.53	6.55	
4	0.5004	49.89	6.77	
5	0.5011	51.55	7.00	
6	0.5021	48.53	6.57	
7	0.5022	48.51	6.56	
Raw material moisture – 6.26%				

Table 5

RESULTS OF THE EVALUATION OF THE "INTRA-LABORATORY PRECISION" PARAMETER IN THE VALIDATION OF THE METHOD OF QUANTITATIVE CONTENT OF ARBUTIN IN COWBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES BY CAPILLARY ELECTROPHORESIS

Content of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves, %			
Repeat	Sample 1 (Irkutsk Region, summer 2019)	Sample 2 (KBR, autumn 2019)	Sample 3 (Altai Krai, autumn 2019)
Executor 1			
1	9.42	6.99	7.17
2	10.10	6.67	6.77
3	10.57	6.55	6.52
Executor 2			
4	9.74	6.77	6.37
5	10.30	6.99	6.73
6	9.50	6.57	6.40
Average value	9.94	6.76	6.66
Standard (relative) deviation (RSD %)	4.64	2.91	4.49

to the introduced content and expressed as a percentage (recovery), the average value of recovery, standard deviation, and relative standard deviation were calculated. The content of arbutin in the test solution was 0.0367 mg/ml (36.7 μg /ml) (Table 6).

So, $t_{cal} < t_{tab}$ (P, f), since 0,73<2,31. The method is correct, because it is not burdened with a systematic error.

As a result, it was experimentally proved that the developed method is suitable for the analysis of arbutin by capillary electrophoresis. The developed method was used for the quantitative analysis of the arbutin content in the samples of cowberry (*Vaccinium vitis-idaea* L.) leaves harvested in several regions of the Russian Federation (Table 7).

As one can see from the results in Table 7, the lowest content of arbutin was found in raw materials harvested in the Bryansk region (4.13±0.18%), the maximum is in the Irkutsk region (9.86±0.43%). Based on the results, the limit of the content of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves is not less than 4.0%, which is slightly lower than in the pharmacopoeial monograph of XIV edition. In this regard, we recommend setting a content standard of at least 4%.

The results show that when using UV spectrophotometry, the results are on average 25% higher (the difference in results is from 16% to 28%) than when determining the quantitative content of the arbutin component by the CE method. Apparently, the contribution of auxiliary compounds in the analysis by UV spectroscopy is anticipated. The wide range of results obtained by UV spectroscopy and capillary electrophoresis (16–28%) may be due to the time of harvesting the raw cowberries (before flowering and after fruit maturation),

Table 6

CALCULATED DATA FOR THE "CORRECTNESS" PARAMETER IN THE VALIDATION OF THE METHOD OF QUANTITATIVE CONTENT OF ARBUTIN IN COWBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES BY CAPILLARY ELECTROPHORESIS

Content of arbutin in extract from cowberry (*Vaccinium vitis-idaea* L.) leaves, μg/ml	Added the reference standard (RS) of arbutin		Expected content, μg	Resulted content, μg	Recovery, %	Metrological characteristics
	μl	μg				
22.17	66.6	8.53	30.7	30.81	100.36	R = 100.87 SD = 3.58 RSD = 3.55 t_{cal} = 0.73
22.17	66.6	8.53	30.7	30.84	100.46	
22.17	66.6	8.53	30.7	30.22	98.44	
22.17	124.4	15.93	38.1	38.88	102.05	
22.17	124.4	15.93	38.1	38.04	99.84	
22.17	124.4	15.93	38.1	38.2	100.26	
22.17	189.3	24.23	46.4	46.07	99.29	
22.17	189.3	24.23	46.4	47.31	101.96	
22.17	189.3	24.23	46.4	48.81	105.19	

Table 7

THE QUANTITATIVE CONTENT OF ARBUTIN IN COWBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES HARVESTED IN VARIOUS PLACES OF VEGETATION, DETERMINED BY CAPILLARY ELECTROPHORESIS

Place of vegetation	n	f	$\bar{x}$	S	$S_{\bar{x}}$	t (P, f)	$\Delta\bar{x}$	$\bar{\varepsilon}$
KBR (autumn 2019)	7	6	6.73	0.1971	0.0745	2.45	0.18	2.71
Perm Krai (spring 2018)	7	6	5.83	0.1615	0.0611	2.45	0.16	2.66
Bryansk Region (spring 2018)	7	6	4.13	0.1365	0.0516	2.45	0.13	3.17
Irkutsk Region (summer 2019)	7	6	9.86	0.4689	0.1772	2.45	0.43	4.40
Altai Krai (autumn 2019)	7	6	6.61	0.3021	0.1142	2.45	0.28	4.23
Moscow Region (spring 2019)	7	6	5.97	0.2288	0.0865	2.45	0.21	3.55

which regulates the RD, due to geographical factor and other conditions.

In order to confirm the results obtained using the CE, the same raw material sample (KBR, autumn 2019) was analyzed by HPLC. A typical chromatogram is shown in Figure 5, and a chromatogram of a solution of a standard arbutin sample is shown in Figure 6.

The results of the quantitative determination of the same sample of medicinal raw materials by HPLC, performed in six-fold repetition, are presented in Table 8.

The content of arbutin in the analyzed sample of raw materials was 6.48±0.12%, which is comparable to the results obtained by the CE method.

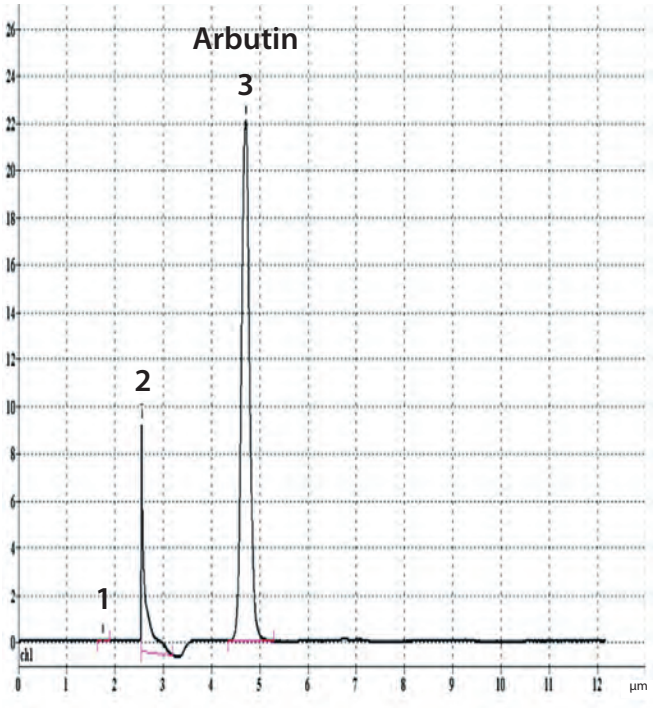

FIG. 5. *Chromatogram of alcohol extraction from cowberry (Vaccinium vitis-idaea L.) leaves*

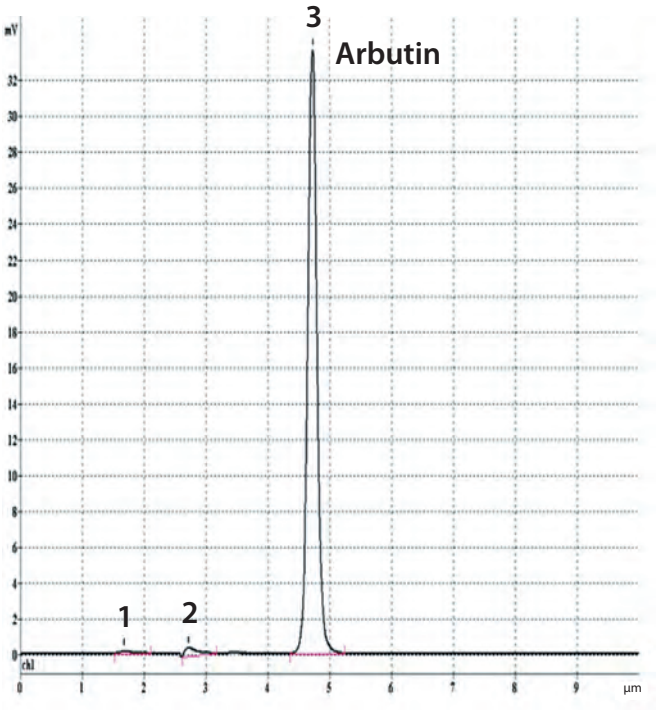

FIG. 6. *Chromatogram of the reference standard of arbutin*

Table 8

RESULTS OF DETERMINATION OF THE QUANTITATIVE CONTENT OF ARBUTIN IN COWBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES BY HPLC METHOD

Experiment No.	Sample weight, g	Arbutin peak area, mV*sec	Content of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves, %	Metrological characteristics
1	0.5020	251.16	6.32	$\bar{x} = 6.48\%$ $S = 0.1109$ $S_{\bar{x}} = 0.0453$ $\Delta\bar{x} = 0.12$ $\bar{x} \pm \Delta\bar{x} = 6.48 \pm 0.12$ $\bar{\varepsilon} = \pm 1.8\%$
2	0.5015	253.97	6.39	
3	0.5004	262.63	6.63	
4	0.5034	260.49	6.53	
5	0.5011	259.08	6.53	
6	0.5021	256.88	6.46	
Raw material moisture – 6.26%; arbutin peak area (0.003216% reference standard solution) 253.97 mB · sec				

CONCLUSION

The content of arbutin in cowberry (*Vaccinium vitis-idaea* L.) leaves from various vegetation sites was determined by the spectrophotometric method. The arbutin content ranged from 5.74% to 11.67%.

The arbutin content determined by capillary electrophoresis varied from 4.13% to 9.86%, which is also confirmed by HPLC data. It was found that overestimated results compared to the separation methods were obtained by the pharmacopoeia method such as UV spectrophotometry. Apparently, this is due to the fact that the UV-spectrophotometry method determines the content of not only the arbutin component, but the total amount of phenol-glycosides. The standard content of arbutin in the cowberry (*Vaccinium vitis-idaea* L.) leaves is determined as at least 4%.

Validation of the method of standardization of cowberry (*Vaccinium vitis-idaea* L.) leaves by capillary electrophoresis was carried out. It was found that the method is linear (R<0.99), precise (RSD<4.64%), and not burdened with a systematic error – the Student's criterion t is $t_{cal} < t_{tab}$ (P, f).

The developed method can be recommended for the analysis of cowberry raw materials.

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