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Development and Validation of A Rp-Hplc Method for The Simultaneous Determination of Luteolin and Apigenin in Herb of *Achillea millefolium* L.

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A method for separation and quantification of two flavonoids by reverse-phase high performance liquid chromatography (HPLC) was developed and validated. Flavonoids present in herb of *Achillea millefolium* L. were analyzed. Luteolin and apigenin were used as calibration standards. The analysis was performed using a Waters X-Terra C18 column (250 x 4.6 mm i.d., 5 µm particle size), as stationary phase, with a flow rate of 1 mL/min and detection at a wavelength of 350 nm. The proposed method was validated by ICH Harmonised Tripartite Guidelines "Validation of analytical procedures: Text and Methodology Q2 (R1)". In this study, an excellent linearity was obtained with r higher than 0.99. Besides, the chromatographic peaks showed good resolution. With other validation data, including precision, specificity, accuracy and robustness, this method demonstrated good reliability and sensitivity, and can be conveniently used for the quantification of luteolin and apigenin in in herb of *Achillea millefolium* L. Further this method can be applied to a standardization of multicomponent herbal remedies, that incorporate *Achillea millefolium* L.

Keyword: *Achillea millefolium* L. Herb, HPLC, Luteolin, Apigenin, Validation.

1. Introduction

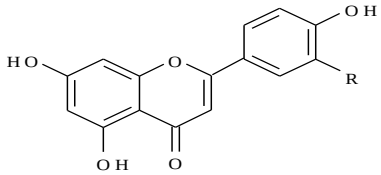
Achillea millefolium L. (yarrow) is recognized as a powerful medicinal plant is widely distributed and has been used medicinally for thousands of years [1]. The medicinal plant raw material from *Achillea millefolium* L. is the herb (Millefolii herba), wich is included in many pharmacopoeias [2].

The diversity and complexity of the phytochemical composition of yarrow species may explain their polyvalent pharmacological activity. The raw material of A. *millefolium* L. contains terpenes, alkaloids and bases, tannins, coumarins, saponins, sterols, vitamins, amino, fatty acids, flavonoids and phenolcarbonic acids [3, 4].

Flavonoids constitute one of the most important groups of pharmacologically active principles in yarrow. It is suggested that anti-inflammatory [3], antimicrobial [5], choleretic [6] and cytotoxic [7] activities of Achillea plants are mainly attributed to the flavonoid complex.

The main components of the flavonoid fraction of yarrow are apigenin and luteolin [2, 8, 9]. Structure of these flavonoids shown in the Fig.1. These flavonoids have a wide spectrum of biological activity.

Apigenin inhibits tumor growth and angiogenesis induced by different cancer cells [10, 11]. It has antiproliferative and anti-tumor properties in pancreas, prostate and colon cancer cells [12, 13, 14].



Luteolin: R= OH
Apigenin: R= H

Fig 1: Chemical structure of luteolin and apigenin.

Luteolin has been shown to possess multiple biological activities such as anti-inflammation, anti-oxidant and anti-cancer [15, 16]. It inhibits the proliferation of many types of cancer cells through regulating the cell cycle [17]. Moreover, luteolin also could induce tumor cells apoptosis including epidermoid carcinoma, leukemia, pancreatic tumor, lung cancer and hepatoma [18].

In our previous studies have demonstrated an ability to use luteolin, as a marker for HPLC standardization of raw materials yarrow in multicomponent herbal remedies [19]. Apigenin was used as a marker of raw materials yarrow for the analysis of multicomponent herbal remedies in other studies [20].

Thus, the objective of this study was to develop and validate a method for the separation and simultaneous quantitative analysis of luteolin and apigenin by HPLC, obtained from an extract of *Achillea millefolium* L. herb.

The method was validated according to ICH Harmonised Tripartite Guidelines "Validation of analytical procedures: Text and Methodology Q2(R1)" [21].

The following validation characteristics were assessed: specificity, linearity, limit of detection and quantification, accuracy, precision and robustness.

2. Materials and Methods

2.1 Plant material

The herb of *Achillea millefolium* L. was collected in the region of Borispol (Kyiv region, Ukraine) in July 2010. Identification of the species was confirmed in State Laboratory for Quality Control of Medicines, State Institution "Institute of pharmacology and toxicology National Academy of Medical Sciences of Ukraine" (Ukraine). A voucher specimen (A011-8) was deposited at the herbarium in this laboratory.

2.2 Chemicals and Reagents.

All reagents and solvents were analytical and HPLC grades (Fluka, USA). Ultra-pure water obtained using a Simplicity® apparatus (Millipore, USA) with conductivity of 0.60 µS/cm was used in all experiments. Luteolin, and apigenin (Fluka, USA) of the highest grade (purity>98.0%) were used as the external standards.

2.3 Instrumentation and Chromatographic Conditions

The analyses were carried out using an HPLC system (Shimadzu, Japan) consisting of a solvent delivery pump (Model LC-20 AD), a diode array detector (Model SPD-20A), an auto-injector (Model SIL-20A) and system controller (Model CBM-20A). Data collection and analyses were performed using LCsolution (ver. 1.22SP1). A gradient elution was performed on a X-Terra C18 column (250 x 4.6 mm i.d., 5 µm particle size) (Waters, USA). The mobile phase consisted of two different solutions, solution A and solution B. Both solutions consisted of acetonitrile and 5% solution of acetic acid, where the proportion of acetonitrile and solution of acetic acid was 90:10 for solution A and 10:90 for solution B. All solutions were degassed and filtered through a 0.45 µm pore size filter (Millipore, USA). Separations were effected by a gradient elution program as follows: from 0 to 10 min, B was isocratic at 80%; from 10 to 16 min, solution B followed a linear change from 80% to 74%; from 16 to 30 min, B was isocratic at 74%; from 30 to 35 min, B linearly changed from 74% to 10%, from 35 to 40 min, B was isocratic at 10%; and from 40 to 55 min, B was isocratic at 80%. The mobile phase flow rate was 1 mL/min and the injection volume was 5 µL. UV detection was performed at 350 nm.

Using these chromatographic conditions, it was possible to confirm the retention time of luteolin and apigenin by injection of each standard separately.

2.4 Sample Preparation

In the present study the extracts of *Achillea millefolium* L. herb were obtained as described in [19].

2.5 Preparation of Standard Solution

Accurately weighed appropriate amounts of the reference compounds (luteolin LUT; apigenin API) were mixed and dissolved in methanol in a 100-mL volumetric flask, to obtain a stock solution. The concentration of the two compounds in this solution was 76.0 µg/mL (API), and 52.5 µg/mL (LUT). Besides, external standards were established at six data points covering the concentration range of each compound according to the level estimated in the plant sample. Working solutions were prepared by stepwise dilution of the stock solution with methanol.

2.5.1 Method Validation

In the validation of the analytical method used for the quantification of luteolin and apigenin in in herb of *Achillea millefolium* L., the following parameters were determined: specificity, linearity, sensitivity, accuracy, precision and robustness.

2.5.2 Specificity
Specificity is the ability of a method to discriminate between the study analyte(s) and other components in the sample. The specificity was demonstrated by running a procedural blank. In addition, the resolution between the peaks of the main flavonoids that could be found in extracts of *Achillea millefolium* L. herb was determined by analysis of chromatograms of the standard solution and the sample solution.

2.5.3 Linearity
The linearity between peak area and concentration was analyzed using two calibration curves obtained with standard solutions at fsix different concentrations of each standard API, and LUT. The concentrations of the two compounds in the solution that was considered 100% was 4.20 µg/mL (LUT), and 3.04 µg/mL (API). The other concentration levels used to construct calibration curves were 10%, 50%, 75%, 100%, 125%, 150 % and 200% of the concentration mentioned above.The data for peak area versus drug concentration were treated by linear regression analysis.

2.5.4 Sensitivity
The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were determined from the calibration curves of the LUT and API standards. *LOD* was calculated according to the expression DPx3/IC, where DP is the standard deviation of the response and IC is the slope of the calibration curve. LOQ was established by using the expression DP x10/IC [21].

2.5.5 Accuracy
The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the LUT and API standard to the sample, at three different levels (5%, 10% and 15%) of the initial concentration of the sample. Each solution was injected in triplicate. Average recoveries were calibrated by the formula recovery (%) = {(amount found - original amount)/amount spiked} x 100.

2.5.6 Precision
The precision of the method was investigated with respect to repeatability, intermediate precision (inter-day variation) and reproducibility by determination of standard solution at 100% of the test concentration. To assess the intra-day precision (repeatability) of the method, the sample was injected six times within a day. The inter-day precision was determined with the sample assayed on different days and by another analyst. Precision was expressed as the relative standard deviations (% RSD) of the concentrations of each compound, LUT and API.

2.5.7 Robustness
Three sample solutions were prepared and analyzed under the conditions established and by changing the wavelength parameter from 348 nm to 352 nm, by using columns from different suppliers and by changing the mobile phase composition (±5% change organic solvent and and (±5% change acetic acid concentration) [1A].

2. Statistical Analysis
The data were submitted to statistical analysis using Excel® software.

3. Results and Discussion
The HPLC method carried out in this study was aimed at developing a chromatographic system, capable of eluting and resolving flavonoid compounds in herb of *Achillea millefolium* L. In the development of the HPLC method for determination of apigenin and luteolin in herb of *Achillea millefolium* L., several solvent systems (methanol-water-acetic acid, acetonitrile-water-acetic acid, tetrahydrofuran-water-acetic acid) and separation columns Waters X-Terra C18 column (250 x 4.6 mm i.d., 5 µm particle size), Waters X-Terra C8 column (250 x 4.6 mm i.d., 5 µm particle size), Supelco Discovery®HS C18 (250 x 4.6 mm i.d., 5 µm particle size), Macherey_Nagel Nucleosil 100-5 C18 (250 x 4.6 mm i.d., 5 µm particle size) were evaluated and compared. The Waters X-Terra C18 column provided better separation of the plant extract than with other specifications or brands of columns.

The choice of detection wavelength was determined by performing a screening with 10 ppm of luteolin, major compound, in methanol in a spectrophotometer UV/VIS. The UV spectra were recorded from 220 to 380 nm and exhibited maximum wavelengths at 318 nm and 350 nm. It was carried out an analysis on HPLC with the two wavelengths and which provided better response even for the others compounds (apigenin) was at a wavelength of 350 nm.

The results for quantification of the flavonoids in the sample were 4,385 µg/mL of luteolin and 2,982 µg/mL of apigenin, which means, 0.0205% and 0.0139% of each compound contained in the *Achillea millefolium* L. herb, based on the dried raw, respectively.

System suitability test showed that critical parameters such as retention time, area and number of theoretical plates met the acceptance criteria on all the experimental days (Table 1).

The specificity of the method was evaluated by analysis of blank, standard and sample solution chromatograms (Figure 2). Good separation between the peaks of LUT and API was achieved, with the retention times, 18.4 min for luteolin and 27.0 min for apigenin. Furthermore, the chromatographic peaks showed good resolution (around 18.7 between LUT and API). In relation to asymetry, the peaks showed values 1.037 for LUT and 1.036 for API.

Table 1: System suitability test.

Compound	Parameter	Acceptance	Average	%RSD	Status
Luteolin	Retention time	% RSD < 2	17.14	0.19	Passed
	Peak area	% RSD < 2	41373	1.42	Passed
	No. of Plates	> 3500	20756	2.35	Passed
	Asymmetry factor	< 2	1.037	0.12	Passed
Apigenin	Retention time	% RSD < 2	25.76	0.12	Passed
	Peak area	% RSD < 2	58901	1.25	Passed
	No. of Plates	> 3500	19845	2.83	Passed
	Asymmetry factor	< 2	1.036	0.56	Passed

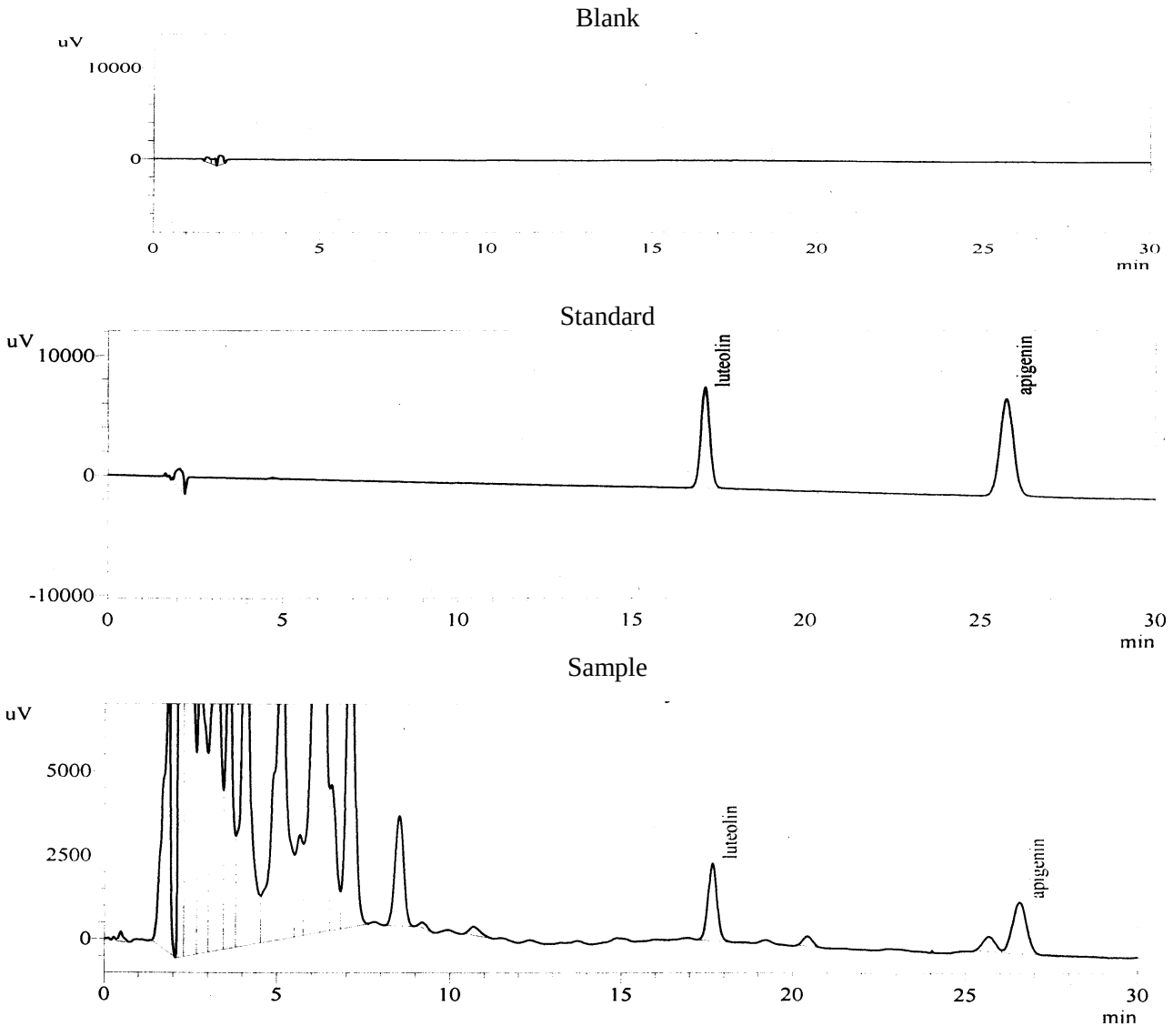


Fig 2: Chromatogram of the blank standard and sample solution performed on Waters X-Terra C18 column (150 x 4.6 mm i.d., 5 µm particle size) at 350 nm.

Linearity was evaluated by the correlation coefficient r, and all values for the three compounds were greater than 0.999, showing that responses for the standard in the concentration ranges examined (from 10 to 200%) were linear. Besides, according to [21], the minimum acceptable correlation coefficient is 0.990.

As shown in Table 2, the LOD values were 0.02 µg/mL for the compounds LUT and API, while the LOQ values were 0.07 µg/mL.

The recovery of the compounds LUT and API was determined by spiking the extracts of *Achillea millefolium* L. herb with known amounts of LUT and API standards. Recovery of each substance was obtained from the calculated amount found and original amount. The results are presented in Table 3 and conform with the recommendations of [21].

The data of the precision are shown in Tables 4, 5. The results display a coefficient of variation less than that recommended by [21] whose limit is 5%.

Table 2: Calibration curve parameters, limit of detection (LOD), limit of quantification (LOQ) for luteolin and apigenin.

Compound	Calibration curve equation	Correlation coefficient (r)	Linear range (µg/mL)	LOD(µg/mL)	LOQ(µg/mL)
Luteolin	y = 14455984x - 1248	0.99960	0.420-8.400	0.02	0.05
Apigenin	y = 14504087x - 2421	0.99918	0.304-6.080	0.02	0.05

Table 3: Results of accuracy determination by analyzing of the luteolin and apigenin of known concentrations.

Compound/Initial concentration	Theoretical concentration after dilution added in the extract (µg/mL)	Amount recovered (µg/mL)	Recovery (%)	Mean (%)	RSD (%)
Luteolin (Concentration measured in the sample = 4,385 µg/mL)	0.225	4.728	102.56	101.54	1.16
		4.694	101.83		
		4.626	100.25		
	0.45	4.769	98.63	100.43	1.66
		4.928	101.92		
		4.871	100.75		
	0.675	5.208	102.93	101.81	0.96
		5.117	101.12		
		5.130	101.38		
Apigenin (Concentration measured in the sample = 2,982 µg/mL)	0.148	3.185	101.75	100.97	1.68
		3.099	99.03		
		3.197	102.14		
	0.296	3.246	99.04	100.99	1.91
		3.372	102.89		
		3.312	101.04		
	0.444	3.512	102.56	100,94	1,40
		3.434	100.23		
		3.427	100.02		

Table 4: Results of the repeatability.

Compound	Mean (µg/mL)±standard deviation (n=6)	RSD (%)
Luteolin	4,385±0.026	0.23
Apigenin	2,982±0.043	0.56

Table 5: Results of the intermediate precision.

Compound	Mean (µg/mL)±standard deviation (n=18)	RSD (%)
Luteolin	4,356±0.038	0.64
Apigenin	2,963±0.068	0.96

Also, there were no significant differences between assay results, indicating that the precision of the proposed method was satisfactory.

Robustness was evaluated to ensure that the HPLC method is insensitive to small changes in the experimental conditions. In this study, the wavelength, column supplier

and pH of the mobile phase were changed. None of the modifications caused any significant change in the resolution or response of the LUT and API peaks.

All results were displayed according to the ICH Harmonised Tripartite Guidelines "Validation of analytical procedures: Text and Methodology Q2(R1)" [21].

The quantitative method developed here was successfully applied in the simultaneous analysis of two different compounds in extracts of *Achillea millefolium* L. herb. Taking into account the results obtained in this study, the proposed method can be conveniently used for the analysis of luteolin and apigenin in extracts of *Achillea millefolium* L. herb.

The proposed method demonstrated high specificity at 350 nm detection for the extracts of *Achillea millefolium* L. herb showing reliability in the quantification of LUT and API. Further, this method can be applied to a standardization of multicomponent herbal remedies, that incorporate *Achillea millefolium* L.

In summary, the method above can be considered specific, exact, precise, linear, robust and easy to perform.

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