Technical paper

Flavonoid Content Assay: Prevalidation and Application on *Plantago L.* Species

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Abstract

This work is aimed to prevalidate and apply UV/Vis spectrophotometric procedure for flavonoid determination in herbal material using $AlCl_3$ as a reagent. Fast and simple, full prevalidation for quality control and standardization of analytical procedure, based on mathematical/statistical testing coupled with system of diagnosis was used to evaluate and demonstrate the reliability of method for flavonoid determination with $AlCl_3$ (*F–Al* procedure). Favourable prevalidation characteristics verified this procedure as a valuable tool in flavonoid analysis, so it was successfully applied for determination of flavonoids in leaves, stems and flowers of *Plantago* L. species growing in Croatia. The results showed the various flavonoid contents between different plant parts (leaves: up to 0.13%; stems: up to 0.07% and flowers: up to 0.07%). The results of flavonoid determination were statistically evaluated by using Principal Component Analysis (PCA) and Student's *t*-test as a contribution to investigation of different taxa of genus *Plantago* L.

Keywords: Prevalidation strategy, flavonoids, aluminium chloride, *Plantago* L.

1. Introduction

Unavoidable part of fundamental and applied investigations in pharmaceutical analysis is analytical procedure with good performance characteristics. The ever-increasing volume of analytical literature concerning quality control requires unambiguously evaluation of the advantages and disadvantages of an analytical procedure.[1–9] For this reason, complete prevalidation,[10] as an informative screening method, should be useful for preliminary evaluation of an analytical process with regard to reasonable need for validation and for systematically obtaining other valuable data. The aim of prevalidation proposal based on peculiar approaches is to diagnose the quality of an analytical procedure and to decide whether a method in question is capable of producing accurate and reliable data. Investigation of dependent and independent variables, as components of analytical system, particularly relationship between them gives insight into the data quality and method's metrological characteristics. Prevalidation is essential to test data validity, e.g. when validate (official) procedure might not exist, when insufficient time would be available for a full validation process, when an analytical method is adopted from some other source or in crisis situations. The efficiency of prevalidation procedure is given by characteristic data such as constants of calibration and analytical evaluation function, standard deviation of procedure, limit of quantitation, and other metrological characteristics.

One part of the present study included application of prevalidation strategy to obtain metrological characteristics and verify spectrophotometric procedure for determination of flavonoids with $AlCl_3$ (*F–Al* procedure). Flavonoids are polyphenolic compounds that occur ubiquitously in plant tissues in relatively high concentrations as sugar conjugates.[11] They occur mostly in O-glycosidic

form with a number of sugars such as glucose, galactose, rhamnose, arabinose, xylose and rutinose. The flavonoid functions in plants are believed to be as protective agents against UV radiation and also against microorganisms.[12] Flavonoids are of particular importance in the human diet as there is evidence that they act as antioxidants,[13–15] antiviral agents[16] and epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer[17–20] and cardiovascular disease.[21]

Another part of these investigations comprehended application of prevalidated *F–Al* procedure for flavonoid analysis in *Plantago* L. species growing in Croatia. The genus *Plantago* comprises 265 species and has cosmopolitan distribution.[22] Medicinally, *Plantago* species are astringents, demulcents, emollients, expectorants, diuretics, antibacterials and antivirals.[23] Phytochemical investigations of *Plantago* species revealed the presence of iridoids, flavonoids, tannins, triterpenes, saponins, and sterols.[23–26]

The spectrophotometric assay based on aluminium chloride complex formation is one of the most commonly used analytical procedures applied to flavonoid content determination in various plants.[27–30] This procedure includes hydrolysis of glycosides, extraction of total flavonoid aglycones with ethyl acetate and complex formation with $AlCl_3$.[31–33] As there are no literature data concerning spectrophotometric determination of total flavonoids in *Plantago* species, application of *F–Al* procedure was used to provide new information regarding phytochemical characterization of these plant species.

2. Experimental

Experimental comprises protocols for prevalidation of *F–Al* procedure, then extraction and determination of flavonoids in *Plantago* species using prevalidated *F–Al* procedure, as well as multivariate analysis of the obtained results.

2. 1. Apparatus

UV/Vis spectrophotometer Agilent 8453 (Agilent, Germany) with PC-HP 845x UV-Visible System (Agilent, Germany) and 1 cm quartz cells were used for all absorbance measurements.

2. 2. Reagents and Solutions

Pro analysi chemicals, as well as double distilled water were used throughout the work. Acetone (Kemika, Croatia), 25% hydrochloric acid (Kemika) and hexamethylenetetramine (Kemika) were used for the hydrolysis of flavonoide glycoside and plant material extraction. Ethyl acetate (Kemika) was used for aglycone extraction. Aluminium chloride hexahydrate (Kemika) was used as complexing agent. Sodium citrate (Kemika), methanol (Kemika) and acetic acid (Kemika) were used for sample preparation. Filtration of prepared sample solutions was performed by using 0.20 μm Minisart-plus membrane filter (Sartorius AG, Germany).

2. 3. Analytical Standards for Prevalidation

Analyte stock standard solution was prepared by exact weighing of 0.01 g quercetin (Roth, Germany), dissolving in 5% solution of acetic acid in methanol and diluting to 100.0 mL with the same solvent. In adequate volume of standard stock solution of quercetin (2.40, 1.94, 1.45, 0.97, 0.48, and 0.24 mL, corresponding to 0.240, 0.192, 0.144, 0.096, 0.048, and 0.024 mg of quercetin, respectively) 0.5 mL of 0.5% sodium-citrate and 2 mL of aluminium chloride was added. Each solution was made up in 25 mL volumetric flask with 5% acetic acid in methanol. After 45 min, the absorbance at 425 nm of the solution was measured. Corresponding compensation solution was prepared and measured identically, but without aluminium chloride. Blank solution was prepared and measured identically, but without analyte.

2. 4. Plant Material

Randomly selected samples of wild growing plants of *Plantago* L. species were collected in the western part of Croatia in June 2003: *P. altissima* L. in the Mirna River Basin (the north-west of peninsula Istria) at altitude of 20 m; *P. coronopus* L., *P. lagopus* L., and *P. maritima* L. near Medulin (small town in the south of Istria) at altitude of 29 m; *P. holosteum.* subsp. *depauperata* Pilger between Vodnjan and Bale (villages in the south of Istria) at altitude of 125 m; *P. holosteum* subsp. *scopulorum* (Degen) Horvatić on the islands of Cres and Lošinj, near small town Osor, at altitude of 10 m; *P. argentea* Chaix and *P. holosteum* Scop. subsp. *holosteum* on the pass Gornje Jelenje (continental part of the West Croatia) at altitude of 880 m.

All plant samples were identified at the Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. Voucher specimens (No. 0071–0078) are deposited in the Herbarium of the Department of Pharmacognosy (Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia). Air-dried samples of leaves, stems, and flowers were phytochemically investigated.

2. 5. Execution of Prevalidation

Standardized measurements were based on a set of 24 blocks of data (6 sets of 4 experiments each) to relate measured values to blank values. Samples were measured in standard working range of one power of ten, alternately in the following group sequence: 1, 6, 2, 5, 3, and 4 (Table 1).

Table 1: Strategy of prevalidation measurements on standardized basis (N = 24 measurements).

Measurements as a process of obtaining results	
Type of measurements	Blank measurements (B), gross measurements (y)
Number of analytical groups	J = 6, j = 1, 2, ..., 6
Group volume	I = IV, i = I, ..., IV
Total number of measurements	$N = J \times I = 24$, n = 1, 2, …, 24
Analyte amount (quercetin)	x (mg)
Analyte working range	1.0 $x_U = x_1 = x_U$ = 0.240 mg, upper level of analyte, 0.8 $x_U = x_2$ = 0.192 mg, 0.6 $x_U = x_3$ = 0.144 mg, 0.4 $x_U = x_4$ = 0.096 mg, 0.2 $x_U = x_5$= 0.048 mg, 0.1 $x_U = x_6 = x_L$ = 0.024 mg, lower level of analyte
Sequence of groups measurements	1, 6, 2, 5, 3, 4
Measure (net signal)	$S = y - B$
Gross signal	y
Blank signal	B

Starting data used for mathematical/statistical evaluation were absorbances obtained by measurement of blanks, standards and compensation solutions. Because of possible influence of quercetin on absorbance of quercetin-$AlCl_3$ complex, prior to mathematical/statistical testing gross signal is corrected with absorbance obtained in measurement of corresponding compensation solution. Compensation solution contained the same quantity of quercetin and other components as corresponding analyte solution except aluminium chloride. Systematic prevalidation strategy and all algorithms together with system of diagnosis were quoted gradually in the paper.[10]

Mathematical/statistical testing comprised descriptive and prognostic statistics. Arithmetic means, standard and relative standard deviations were used for characterization of all analytical groups (1–6). Prognostic statistics included: checking of groups 1 and 6, testing of data homogeneity, estimation of calibration and analytical evaluation function, outlier recognition and estimation of limiting values. The application of expert system to evaluation of spectrophotometric procedure for determination of quercetin with aluminium chloride was presented in section *Results and Discussion* in Tables 2–9. Test statistic values were referred to as requirements R throughout the paper.

2. 6. Extraction and Determination of Total Flavonoids

The content of total flavonoids (quercetin type) in *Plantago* species was determined by *F–Al* procedure (by using method according to Christ and Müller).[31] Powdered plant material (0.20 g of each leaves, stems and flowers) was extracted with 20 mL of acetone, 2 mL of 25% HCl and 1 mL of 0.5% hexamethylenetetramine (boiling water bath, 30 min). Each extract was filtered and extraction of the same herbal material was repeated three times with 20 mL of acetone (boiling water bath, 10 min). After cooling and filtration each extract was made up to 100.0 mL with acetone (basic sample solution, BSS). 20 mL of BSS was mixed with 20 mL of water and then extracted with ethyl acetate (first with 15 mL and then three times with 10 mL). Ethyl acetate extracts were rinsed two times with water then filtered and made up to 50.0 mL with ethyl acetate (Solution 1, S1). In 10 mL of S1 0.5 mL of 0.5% solution of sodium citrate and 2 mL of $AlCl_3$ (2 g of $AlCl_3$ in 100 mL of 5% acetic acid in methanol) were added and then made up to 25.0 mL with 5% methanolic solution of acetic acid (sample solution, SS). The same procedure was performed with blank sample solution but without $AlCl_3$. After 45 minutes, yellow solutions were filtered and absorbance at 425 nm was measured. The content of total flavonoids was evaluated upon three independent analyses. The yield was calculated as quercetin toward following expression

$$\% = A \times 0.772 / b,$$

where A is absorbance and b represents mass of dry herbal material in grams.

2. 7. Statistical Analysis

The results of flavonoid analysis were evaluated using Student's t-test and multivariate analysis.[34–36] The Principal Component Analysis (PCA) calculation was based on the correlation matrix between the values of the characteristics, which means that the contribution of each variable was independent of the range of its values.[37–39] The statistical analysis of the results of flavonoids determination was performed using software Statistica 6.0.

3. Results and Discussion

3. 1. Analysis of Prevalidation Results

The analytical signal y, proportional to the absolute mass of the quercetin present, as well as signals obtained from compensation and blank solutions were transformed into the corresponding absorbance values which were used for calculation. Starting data were: mass of quercetin, x, within the working range from 0.024 to 0.240 mg,

absorbances obtained in measurements of the blank (*B*), the sample (*y*) as well as calculated neto absorbance (*S*).

3. 2. Characterization of Groups 1 to 6

Standardized measurements and calculated values of *F–Al* procedure were given in Table 2. Standard deviation or relative standard deviation values obtained for all kind of absorbances in each experimental group were used as a measure of precision.[8, 40] Reasonable precision in accordance with prevalidation criteria[41] was attained for the absorbances obtained in measurements of the sample (s_{ry} from ± 0.72 to ± 6.57) and for corrected absorbances (s_{rS} from ± 0.65 to 7.22). Reasonably, the highest values of relative standard deviation, as a reference to reduced precision, were obtained in the group with the smallest analyte content, x_6. It is obvious from the results that fluctuations obtained in the measurement of blank samples effect the lower level of precision (s_{rB} from ± 14.44 to ± 30.59) and influence on the quality of results could be expected. In the case of great fluctuations of blanks, the influence of blanks could be neglected only if they are small enough in relation to gross values. Therefore, additional checking is necessary to conclude about this type of influences (see R7 and R8, Table 4).

3. 3. Checking of Limiting Groups 1 and 6

The preliminary check of working range-limiting groups represented quality control of measurement in a group with the smallest mass of analyte and enables unambiguous distinction between gross and blank signal at x_6 (R1, Table 3). Applicability of this requirement was also extended to the recognition of influence of blank values dispersion on the standard deviation of the procedure (s_M) through heuristic requirement R2 (Table 3). Preliminary information obtained in this evaluation showed that determination limit is expected below x_6 (R3, Table 3). For the standard measurement, requirement that s_r values for both gross and corrected measurements at x_U and x_L lie below ± 2.5 and ± 25%, respectively was satisfied (R3, Table 3). For the *F–Al* system under study gross signals could be clearly distinguished from blank signals at x_6, although high values of standard deviation of blanks were obtained. Furthermore, total s_r value for blanks is 27.8% which corresponds to prevalidation acceptance criteria (total s_r < ± 50%).[10]

Table 2: Standard measurements for *F–Al* procedure.

Group (*j*)	Sample No. (i)	x[a], (mg)	*B*	$\bar{B}$ / s_B / s_{rB},%	*y*	$\bar{y}$ / s_y / s_{ry},%	*S*	$\bar{S}$ / s_s / s_{rS},%	A[b]	$\bar{A}$ / s_A / s_{rA},%
1	I	0.240	0.0043	0.0043/± 0.0009/	0.6999	0.6942/± 0.0050/	0.6956	0.6899/± 0.0049/	2.8983	2.8745/± 0.0187/
	II		0.0056	± 21.40	0.6969	± 0.72	0.6913	± 0.65	2.8804	± 0.65
	III		0.0036		0.6900		0.6864		2.8600	
	IV		0.0037		0.6899		0.6862		2.8592	
6	I	0.024	0.0032	0.0037/± 0.0005	0.0697	0.0643/± 0.0042/	0.0665	0.0605/± 0.0044/	2.7708	2.5219/± 0.1820/
	II		0.0034	± 14.44	0.0594	± 6.57	0.0560	± 7.22	2.3333	± 7.22
	III		0.0039		0.0637		0.0598		2.4917	
	IV		0.0044		0.0642		0.0598		2.4917	
2	I	0.192	0.0055	0.0041/± 0.0013/	0.5604	0.5566/± 0.0047/	0.5549	0.5525/± 0.0042/	2.8901	2.8777/± 0.0022/
	II		0.0045	± 30.59	0.5521	± 0.84	0.5476	± 0.77	2.8521	± 0.77
	III		0.0039		0.5609		0.5570		2.9010	
	IV		0.0025		0.5531		0.5506		2.8677	
5	I	0.048	0.0030	0.0028/± 0.0006/	0.1175	0.1230/± 0.0062/	0.1145	0.1202/± 0.0062/	2.3854	2.5037/± 0.1299/
	II		0.0020	± 20.10	0.1220	± 5.06	0.1200	± 5.19	2.5000	± 5.19
	III		0.0033		0.1206		0.1173		2.4438	
	IV		0.0030		0.1319		0.1289		2.6854	
3	I	0.144	0.0043	0.0051/± 0.0011/	0.4060	0.4097/± 0.0074/	0.4017	0.4047/± 0.0076/	2.7896	2.8101/± 0.0530/
	II		0.0058	± 21.43	0.4186	± 1.81	0.4128	± 1.88	2.8667	± 1.88
	III		0.0062		0.4017		0.3955		2.7465	
	IV		0.0040		0.4126		0.4086		2.8375	
4	I	0.096	0.0046	0.0035/± 0.0010/	0.2807	0.2766/± 0.0071/	0.2761	0.2731/± 0.0063/	2.8760	2.8451/± 0.0659/
	II		0.0040	± 28.19	0.2812	± 2.57	0.2772	± 2.31	2.8875	± 2.31
	III		0.0024		0.2661		0.2637		2.7469	
	IV		0.0030		0.2785		0.2755		2.8698	
6 groups mean $\bar{s}$ ($\bar{s}_r$, %)			± 0.0009 (± 23.31)		± 0.0059 (± 3.65)		± 0.0057 (± 3.85)			± 0.0983 (± 3.85)

[a] Mass of quercetin
[b] Measure of particular sensitivity, $A_n = S_n/x_n$

Table 3: Checking of limiting groups 1 and 6.

Requirement No.	Result	Diagnosis
R1	$AC = 17.25$	Significant influence of blank dispersions on s_M is not expected
R2	$R = 162.48\%$	
	$s_{rB1} = \pm 21.40\%$	
	$s_{rB6} = \pm 14.44\%$	
R3	$s_{ry6} = \pm 6.57\%$	Determination limit is expected below x_6
	$s_{rS6} = \pm 7.22\%$	
	$s_{ry1} = \pm 0.72\%$	
	$s_{rS1} = \pm 0.65\%$	
	$L_{DG} = 0.0139$ mg	
	$\bar{L}_{DG} = 0.0028$	
	$s_{rL} = \pm 25.25\%$	
R4	$R = 12.71$	Very good resolution of signals
R5	$R = 3.85$L	inear calibration function is not expected

Additional checking of quality of signal resolution for the *F–Al* procedure showed that gross and blank signals were very good distinguished (R4, Table 3). The preliminary linearity check was applied to *A* values (particular sensitivity values, $A = S/x$) for limiting groups 1 and 6. Although the obtained value (R5, Table 3) was very close to tabulated value t (3.707) for this requirement, linear calibration function is not expected. Since only two limiting groups were included in this requirement, systematic and deep evaluation is unavoidable.

3. 4. Testing of Data Homogeneity

Analysis of variance applied to the 6 groups of blank values in *F–Al* procedure indicated homogeneity of blank values (R6, Table 4). The influence of blank values is almost negligible because they are small enough in relation to information obtained at the upper analyte level (R7, Table 4) and total standard deviation of blank values (s_{rBN}) was not exceeded ± 50% (R8, Table 4). Since requirements R6, and/or R7, and R8 were satisfied, influence of blank values on results could be excluded and each y value could be corrected with grand blank mean ($\bar{B}_N$) in *F–Al* procedure.

Table 4: Testing of data homogeneity.

Requirement No.	Result	Diagnosis
R6	$s^2_{Bb} = 2.34 \times 10^{-6}$	Homogeneous blank values
	$s^2_{Bw} = 8.65 \times 10^{-7}$	
	$R = 2.71$	
R7	$\bar{B}_N$ should be < 0.0035	Influence of blank value is not negligible
	$\bar{B}_N = 0.0039$	
R8	$s_{rBN} = \pm 27.77$	
	$s_{BN} = 1.09 \times 10^{-3}$	
R9	$R(s_B) = 2.85$	s.h.[a]
	$R(s_{rB}) = 1.78$	s.h.
	$R(s_y) = 1.41$	s.h.
	$R(s_{ry}) = 17.12$	a.h[b]
	$R(s_S) = 1.63$	s.h.
	$R(s_{rS}) = 19.60$	a.h.
	$R(s_A) = 17.42$	a.h.
	$R(s_{rA}) = 19.60$	a.h.

[a]s.h. – strongly homogenous
[b] a.h. – almost homogenous

Barttlet test, applied to s and s_r values for B, y, S, A values (R9, Table 4), as well as to the values of the apparent mass of analyte, $\hat{x}$ (Table 8) provides an insight into the data structure and enables quick recognition of the source of error. For the *F–Al* procedure under study, Barttlet test was pointed to high data homogeneity of standard and relative standard deviations for majority of values. Lower level of homogeneity was attained for relative standard deviations of gross signals which influence lower homogeneity of neto signals (R9, Table 4).

3. 5. Relation Between Signal and Concentration

The characteristic data evaluated by preliminary inspection of the relationship between signal values and content of analyte (method of the least squares) were: determination coefficient (r^2), slope of a line (b), intercept of a line (a), errors in the slope (s_b), and errors in intercept (s_a) (R10, Table 5). The position of the grand mean of sig-

Table 5: Quality of relationship analyte amount – analytical signal.

Analyte-signal relationship		
Requirement No.	**Result**	**Diagnosis**
R10	r = 0.99957	
	b = 2.9354	
	a = – 0.0139	
	$s_y = \pm 0.00104$	
	$s_b = \pm 0.08630$	
	$s_a = \pm 0.00041$	
	centroid = (0.4133, 0.3503)	
R11	$R = 159.54$	Significant correlation
R12	$\pm C_b = 2.9354 \pm 0.08630$	
	$\pm C_a = -0.0139 \pm 0.00041$	
t-testing for reality of calibration constants		
R13	$V = 2.85$	
	$R_V = 203.24$	
	$s_V = \pm 0.01405$	
	$s_m = \pm 0.0100$	Ideal calibration function
	$\hat{S} = 2.85x$	
t-testing for reality of analytical evaluation constants		
R14	$V = 0.35$	
	$R_V = 203.24$	
	$s_V = \pm 0.00172$	
	$s_M = \pm 0.0035$	Ideal analytical evaluation function
	$\hat{x} = 0.35S$	

nal values $\overline{S}_N$, and the grand mean of mass of analyte $\overline{x}_N$, is known as the *centroid* of all the points. Significance of determination coefficient using statistical *t*-test showed that for the *F–Al* procedure significant correlation does exist (R11, Table 5). Errors in the slope and intercept of the regression line were used to estimate confidence limits for the slope and intercept (R12, Table 5).

Since the method of the least squares *a priori* assumed a linear relationship between analytical signal and analyte content, complete and deep evaluation of calibration function using standardized mathematical/statistical procedure was performed.[10] The characteristic data evaluated from this procedure were the constants of the calibration and analytical evaluation function, the mean errors of the constants and the standard deviation, s_M, of the analytical procedure in the given working range (R13 and R14, Table 5). For the system under study, both ideal calibration and analytical evaluation functions were found. From the final calibration and analytical evaluation function it was possible to evaluate apparent signal values, $\hat{S}$, and apparent masses of analyte, $\hat{x}$, respectively. Furthermore, analytical functions were used for recognition of outliers and evaluation of analyte limiting values.

3. 6. Outlier Recognition

Testing for the outlier was done by comparison of $|S^*|$ and $|x^*|$ values with the *t*-values of confidence intervals for P = 95 and 99% confidence level.[10] According to prevalidation acceptance criteria, one outlying value is tolerable within the 24-data population. Since one outlying value is observed in *F–Al* procedure, there is no objection on the homogeneity of the data material (R15, Table 6).

Table 6: Test for outliers.

Requirement No.	Result	Diagnosis
R15	$\lvert S^*_{13}\rvert > 2.069$	One outlying value, No objection on data material
	$\lvert x^*_{13}\rvert > 2.069$	One outlying value, No objection on data material

3. 7. Estimation of Limiting Values

According to Gottschalk approach,[42] calculation of L_{DG} was based on s_M value of analytical evaluation function and for the *F–Al* procedure gives the value of L_{DG} = 0.0139 mg of quercetin. This calculated value being bellow the respective x_6 level confirmed the correctness of preliminary test R3 (Table 3). According to[3, 41] limit of detection and related quantities comprise the slope of the analytical calibration function (sensitivity), *V*, the total standard deviation of blank values, s_{BN}, and *k* stands for suggested numerical factor of 3.3 and 10 for the limit of detection, L_D, and limit of quantitation, L_Q, respectively. All estimated limiting values were significantly lower then the mass of quercetin at lower analyte level, x_6 (R16, Table 7).

Table 8: Data structure for *F–Al* procedure.

j	I	S	$\hat{S}$	ΔS	S^*	x	$\hat{x}$	$\hat{\overline{x}}$	$s_{\hat{x}}$	$s_{r\hat{x}}$, %	Δx	$\Delta x/x \times 100$, %	$\overline{\Delta x}$	$\overline{\Delta x}/x \times 100$, %	x^*
1	I	0.6960	0.6851	– 0.0105	1.0499	0.240	0.2436	0.2416	± 0.002	± 0.65	+ 0.0036	+ 1.48	+ 0.0016	+ 0.64	1.0115
	II	0.6930		– 0.0062	0.6208		0.2420				+ 0.0020	+ 0.85			0.5826
	III	0.6861		– 0.0013	0.1319		0.2403				+ 0.0003	+ 0.14			0.0938
	IV	0.6860		– 0.0011	0.1120		0.2403				+ 0.0003	+ 0.11			0.0739
6	I	0.0665	0.0685	+ 0.0020	0.2003	0.024	0.0233	0.0212	± 0.002	± 7.22	– 0.0007	– 2.98	– 0.0028	– 11.70	0.2041
	II	0.0560		+ 0.0125	1.2480		0.0196				– 0.0044	– 18.30			1.2515
	III	0.0560		+ 0.0087	0.8689		0.0209				– 0.0031	– 12.76			0.8724
	IV	0.0598		+ 0.0087	0.8689		0.0209				– 0.0031	– 12.76			0.8724
2	I	0.5549	0.5481	– 0.0068	0.6822	0.192	0.1943	0.1935	± 0.001	± 0.77	+ 0.0023	+ 1.19	+ 0.0015	+ 0.76	0.6516
	II	0.5476		+ 0.0005	0.0461		0.1917				– 0.0003	– 0.14			0.0766
	III	0.5570		– 0.0089	0.8918		0.1950				+ 0.0030	+ 1.57			0.8611
	IV	0.5506		– 0.0025	0.2532		0.1928				+ 0.0008	+ 0.41			0.2227
5	I	0.1145	0.1370	+ 0.0225	2.2466	0.048	0.0401	0.0421	± 0.002	± 5.19	– 0.0079	– 16.48	– 0.0059	– 12.34	2.2536
	II	0.1200		+ 0.0170	1.6978		0.0420				– 0.0060	– 12.47			1.7049
	III	0.1173		+ 0.0197	1.9672		0.0411				– 0.0069	– 14.44			1.9743
	IV	0.1289		+ 0.0081	0.8098		0.0451				– 0.0029	– 5.98			0.8172
3	I	0.4017	0.4115	+ 0.0094	0.9326	0.144	0.1407	0.1417	± 0.003	± 1.88	– 0.0034	– 2.33	– 0.0023	– 1.61	0.9552
	II	0.4128		+ 0.0018	0.1749		0.1445				+ 0.0005	+ 0.37			0.1521
	III	0.3955		+ 0.0156	1.5512		0.1385				– 0.0055	– 3.84			1.5736
	IV	0.4086		+ 0.0025	0.2441		0.1431				– 0.0009	– 0.65			0.2669
4	I	0.2761	0.2740	– 0.0021	0.2064	0.096	0.0967	0.0956	± 0.002	± 2.31	+ 0.0007	+ 0.70	– 0.0004	– 0.39	0.1911
	II	0.2772		– 0.0032	0.3162		0.0971				+ 0.0011	+ 1.10			0.3009
	III	0.2637		+ 0.0103	1.0308		0.0923				– 0.0037	– 3.82			1.0458
	IV	0.2755		– 0.0015	0.1465		0.0965				+ 0.0005	+ 0.48			0.1313

Barttlet test for $\overline{x}$: $R(s)$ = ± 1.63, s.h.; $R(s_r)$ = ± 19.60, a.h., Six groups mean for $\hat{x}$: $\overline{s}$ = ± 0.0020; $\overline{s}_r$ = ± 3.85%

Table 7: Estimation of limiting values.

Requirement No.	Result	Diagnosis
R16	Ideal calibration function	
	$\hat{S} = 2.85x$	
	$S_D = 0.0072$	S_D is expected below S_6
	$L_D = 0.0011$ mg	
	$L_Q = 0.0038$ mg	L_Q is expected below x_6

Analysis of variance, the Barttlet test, reality of linear analytical evaluation function and agreement of actual, x, and appropriate, $\hat{x}$ values gave information on quality of the analytical procedure. With defined analytical evaluation function, it was possible to conclude on accuracy as a total error of analytical procedure using random deviations as well as absolute and relative systematic deviations. The data structure for the *F–Al* procedure is given in Table 8. The procedure was characterized by L_Q value of 0.0038 mg of quercetin and by systematic deviations ranging from – 12.34% to + 0.76%. It is likely that small deviations of blank and gross values are the principal generator of random deviations ranging from ± 0.65% to ± 7.22%. The extensive prevalidation metrological characteristics are summarized in Table 9.

3. 8. Quantitative Analysis of Total Flavonoids in *Plantago L.* Species

The yields of total flavonoids in leaves, stems, and flowers of *Plantago* species are given in Table 10. The results of *F–Al* procedure showed that generally the highest content of flavonoids was observed in leaves, compared to stems and flowers. The yields of flavonoids in leaves varied from 0.053% (*P. coronopus*) to 0.131% (*P. maritima*). The maximum flavonoid concentration in stems was 0.065% (*P. holosteum* subsp. *depauperata*), while the smallest amount contained sample of *P. coronopus* (0.008%). Flowers of *P. argentea* (0.067%) were the most abundant with flavonoids, while the minimum concentration was determined in flowers of *P. maritima* (0.007%).

Generally, the highest content of total flavonoids was determined in above-ground parts (leaves + stems + flowers) of *P. argentea* (0.221%), while the lowest amount

Table 9: Prevalidation characteristics of *F–Al* procedure for quercetin determination.

Parameter	F–Al procedure					
Working range [mg]	0.240 – 0.024					
Information value range [absorbance units]	0.6999 – 0.0594					
Analyte-signal relationship	$r = 0.9996$					
Calibration function	$\hat{S} = 2.85\ x$					
Analytical evaluation function	$\hat{x} = 0.35\ S$					
Standard deviation of procedure	± 0.0035					
Limit of detection, L_D [mg]	0.0011					
Limit of quantitation, L_Q [mg]	0.0038					
Groups data						
Actual [mg]	0.240	0.192	0.144	0.096	0.048	0.024
Found [mg]	0.244	0.194	0.141	0.097	0.040	0.023
Random deviations						
$s_{\bar{x}}$ [mg]	± 0.002	± 0.001	± 0.003	± 0.002	± 0.002	± 0.002
$s_{r\bar{x}}$, [%]	± 0.65	± 0.77	± 1.88	± 2.31	± 5.19	± 7.22
Systematic deviations, $\Delta\bar{x}$						
Absolute [mg]	+ 0.0016	+ 0.0016	– 0.0023	– 0.0004	– 0.0059	– 0.0028
Relative [%]	+ 0.64	+ 0.76	– 1.61	– 0.39	– 12.34	– 11.70

Table 10: Content of total flavonoids in different plant organs of *Plantago* L. species.

Plant	Total flavonoids (%); $\bar{X}$ ± SD, $n = 3$		
	Leaves	Stems	Flowers
P. altissima	0.095 ± 0.017	0.013 ± 0.001	0.024 ± 0.001
P. argentea	0.110 ± 0.037	0.044 ± 0.002	0.067 ± 0.002
P. coronopus	0.053 ± 0.015	0.008 ± 0.001	0.025 ± 0.002
P. holosteum subsp. *depauperata*	0.115 ± 0.019	0.065 ± 0.003	0.039 ± 0.002
P. holosteum subsp. *holosteum*	0.093 ± 0.019	0.047 ± 0.002	0.032 ± 0.002
P. holosteum subsp. *scopulorum*	0.065 ± 0.018	0.049 ± 0.002	0.011 ± 0.001
P. lagopus	0.094 ± 0.019	0.038 ± 0.001	0.026 ± 0.005
P. maritima	0.131 ± 0.011	0.038 ± 0.001	0.007 ± 0.001

of the examined compounds was established for the above-ground parts of *P. coronopus* (0.089%).

3. 9. Mathematical/statistical Evaluation of Flavonoid Analysis in *Plantago L.* Species

As regard to content of total flavonoids in leaves, stems and flowers, Principal Component Analysis (PCA) separated investigated *Plantago* species as it is shown on Figure 1.

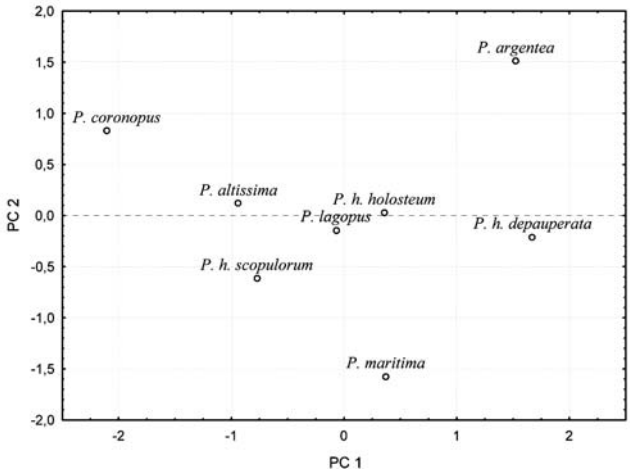

Figure 1: PCA of total flavonoids in *Plantago* L. species.

The most similar species were *P. holosteum* subsp. *holosteum* and *P. lagopus*. Higher degree of separation showed samples of *P. argentea*, *P. holosteum* subsp. *depauperata* and *P. maritima*. These three species had the highest contents of total flavonoids. Above-ground parts of *P. coronopus* contained the smallest amount of examined compounds, so it was also significantly separated from the species in the central part of the PCA scatterplot.

The first principal component explains 53.76% of the total variance, the second one 28.14% and the third component explains 18.10% of the variance. Eigen-vectors matrix with the loading of each variable in each principal component is presented in Table 11. The highest contribution to the first PC axis gave the content of flavonoids in stems and leaves. The content of flavonoids in flowers contributed the most to the second PC axis.

Table 11: Eigen-vectors of the principal components.

Variable	PC 1	PC 2	PC 3
Flavonoids (leaves)	0.624900	–0.360836	0.692313
Flavonoids (stems)	0.636767	–0.277492	–0.719393
Flavonoids (flowers)	0.451694	0.890391	0.056364

The results of *F–Al* procedure were also evaluated by using Student's *t*-test (Table 12) in order to illustrate various distributions of total flavonoids in different plant organs of the same plant species.

The greatest difference was established for the specimens of *P. maritima*, while the statistically insignificant difference was obtained for *P. holosteum* subsp. *scopulorum*. In general, statistically significant differences were observed between flavonoid content in stems and flowers of the same species ($p < 0.001$, except in *P. lagopus*: $p < 0.02$).

4. Conclusions

Very simple, useful, and informative prevalidation concept for quality control and standardization of analytical procedure was used to obtain prevalidation characteristics of procedure for spectrophotometric determination of flavonoids with $AlCl_3$. Good metrological characteristics obtained for *F–Al* procedure confirmed the usefulness of the system under study which is characterized by both ideal calibration and analytical evaluation functions, very low limit of quantitation ($L_Q = 0.0038$ mg) and favourably random (from ± 0.65% to ± 7.22%) and systematic (from – 12.34% to + 0.76%) deviations accordant with prevalidation acceptance criteria. Favourable prevalidation characteristics confirm the usefulness of *F–Al* procedure as a standard method for flavonoids determination in plant material.

The results of flavonoids analysis in *Plantago* species performed by *F–Al* procedure showed that leaves generally contained the greater amount of flavonoids

Table 12: Statistical comparison of total flavonoid content in different plant organs of investigated *Plantago* L. species using Student's *t*-test.

Plant	Probability (p)		
	Leaf-stem	Leaf-flower	Stem-flower
P. altissima	< 0.010	< 0.010	< 0.001
P. argentea	< 0.050	< 0.200	< 0.001
P. coronopus	< 0.010	< 0.050	< 0.001
P. holosteum subsp. *depauperata*	< 0.020	< 0.010	< 0.001
P. holosteum subsp. *holosteum*	< 0.020	< 0.010	< 0.001
P. holosteum subsp. *scopulorum*	< 0.300	< 0.010	< 0.001
P. lagopus	< 0.010	< 0.010	< 0.020
P. maritima	< 0.001	< 0.001	< 0.001

compared to stems and flowers of investigated plants. The highest concentration was determined in leaves of *P. maritima* (0.131%), while the highest content in stems and flowers were obtained for the specimens of *P. holosteum* subsp. *depauperata* (0.065%) and *P. argentea* (0.067%), respectively. The highest total flavonoid content in above-ground parts had *P. argentea* (0.221%), while the lowest amount was determined in the above-ground parts of *P. coronopus* (0.089%).

Multivariate analysis (PCA) of total flavonoids in *Plantago* species showed that the most similar species were *P. holosteum* subsp. *holosteum* and *P. lagopus*. PCA also pointed out species: *P. holosteum* subsp. *depauperata*, *P. argentea* and *P. maritima*, with the highest amount of total flavonoids.

Student's *t*-test revealed differences in distributions of total flavonoids in different plant organs of the same plant species. The greatest difference was established for the specimens of *P. maritima* and the lowest for *P. holosteum* subsp. *scopulorum*.

Ultimately, the present study showed that the prevalidation strategy has proven valuable for evaluating the validity of *F–Al* procedure, which was successfully applied for flavonoids determination in plant material. Moreover, the obtained results of the performed analytical procedure and statistical analysis have contributed to the investigation of the complex genus *Plantago*.

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Povzetek

V delu je opisan postopek validacije in uporaba spektrofotometrične metode UV/Vis z aluminijevim trikloridom kot reagentom za določevaje flavonoidov v rastlinskih vzorcih. Za kontrolo kakovosti in standardizacijo analiznega postopka ter potrditev zanesljivosti metode je bila izvedena hitra in enostavna validacija na osnovi matematično-statističnega testiranja. Ugodni parametri validacije so potrdili primernost metode za določevanje flavonoidov z $AlCl_3$ (F-Al metoda), ki je bila tudi uspešno uporabljena za določevanje flavonoidov v listih, steblih in cvetovih trpotcev *Plantago* L. Rezultati so pokazali različno vsebnost flavonoidov v različnih deli rastlin vzorčenih na Hrvaškem (listi: do 0,13 %; stebla: do 0,07 % in cvetovi: do 0,07 %). Rezultati določevanja flavonoidov so bili za proučevanje različnih taksonov iz rodu *Plantago* L. statistično ovrednoteni z metodo analize glavnih osi in Studentovega t-testa.