Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 837–842
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Spectrophotometric method for polyphenols analysis: Prevalidation and application on *Plantago* L. species

R. Jurišić Grubešić [a], J. Vuković [a,*], D. Kremer [b], S. Vladimir-Knežević [c]

[a] *Department of Analytics and Control of Medicines, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, HR-10000 Zagreb, Croatia*
[b] *Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, Schrottova 39, HR-10000 Zagreb, Croatia*
[c] *Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Marulićev trg 20, HR-10000 Zagreb, Croatia*

Received 29 March 2005; received in revised form 11 May 2005; accepted 11 May 2005
Available online 22 June 2005

Abstract

The prevalidation strategy was applied to evaluate UV–vis spectrophotometric procedure with Folin–Ciocalteu's reagent for polyphenols determination. Favourable prevalidation characteristics verified this procedure as a valuable tool in polyphenols analysis and it was successfully applied for determination of total polyphenols and tannins in leaves, stems and flowers of *Plantago* L. species growing in Croatia. The results showed the variety of total polyphenols content between different plant parts (leaves: up to 10.15%; stems: up to 4.34% and flowers: up to 5.56%). The content of tannins in stems was from 0.28% to 1.00%, while leaves and flowers contained tannins in concentrations of 2.26% and 2.21%, respectively. The results of polyphenols determination were evaluated by using multivariate analysis (UPGMA and PCA) as a contribution to elucidation of relations between different taxa of genus *Plantago* L.

Keywords: Polyphenols; Prevalidation strategy; Folin–Ciocalteu's reagent; *Plantago* L.; Multivariate analysis; Mathematical/statistical evaluation; UPGMA; PCA

1. Introduction

Pharmaceutical analysis requires unambiguously evaluation of the advantages and disadvantages of an analytical procedure/method. Evaluation of optimal working conditions and performance characteristics, ruggedness testing [1–3], validation of procedure [4–9], as well as investigation of relationship between acceptance criteria and validation results are unavoidable parts of investigations of new analytical procedure. Prevalidation proposed by Grdinić and Vuković [10], as original, checked and informative screening method should be useful to prevalidate a new analytical method that has been developed or to verify that an analytical method adopted from some other source is applied sufficiently well. 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, metrological characteristics of the analytical procedure, etc. One part of the present study included application of prevalidation strategy to obtain metrological characteristics and verify spectrophotometric procedure for determination of polyphenols with Folin–Ciocalteu's reagent (*FCR* procedure). Polyphenols represent a large group of structurally related compounds present in many natural products, mainly in fruits and vegetables contributing to their flavour and colour [11]. Natural polyphenols can range from simple molecules such as phenolic acid to large highly polymerized compounds such as tannins. There is an increasing interest in polyphenols due to their potentially positive effect against certain diseases, mainly some forms of cancer and coronary heart diseases. They can act as

* Corresponding author. Tel.: +385 1 49 200 89; fax: +385 1 49 200 89.
E-mail address: jadranka@pharma.hr (J. Vuković).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.05.004

free radical scavengers neutralizing dangerous reactive oxygen species as well as metal ion chelators and both these activities are responsible for antioxidant properties [12]. The polyphenols could also be considered as antimicrobial and anti-inflammatory agents [13].

Another part of these investigations comprehended application of prevalidated *FCR* procedure for polyphenols analysis in *Plantago* L. species growing in Croatia. The genus *Plantago* comprises 265 species and has cosmopolitan distribution [14]. Medicinally, *Plantago* species are astringents, demulcents, emollients, expectorants, diuretics, antibacterials and antivirals [15]. Phytochemical investigations of *Plantago* species revealed the presence of iridoids, flavonoids, tannins, triterpenes, saponins, and sterols [15–18]. Phenolic compounds such as phenylpropanoid glucoside, plantamajoroside, rosmarinic and chlorogenic acid as well as luteolin-7-*O*-monoglucoside were isolated from *Plantago lagopus* L. [19]. As there are no literature data concerning spectrophotometric determination of total polyphenols and tannins in *Plantago* species, application of FCR procedure was used to provide new information regarding phytochemical characterization of these plant species.

2. Experimental work

2.1. Apparatus

UV–vis spectrophotometer Agilent 8453 (Agilent, Germany) with PC-HP 845x UV–vis System (Agilent, Germany) and 1 cm quartz cells was used for all absorbance measurements.

2.2. Reagents and solutions

Pro analysi chemicals, as well as double distilled water were used throughout the work. The solution of 30% methanol (Kemika, Croatia) was used for plant material extraction. The solution of 33% sodium carbonate decahydrate (Kemika, Croatia) was used for sample preparation. Acetate buffer (pH 5.0) was prepared by mixing 1.92 g sodium acetate trihydrate (Kemika, Croatia) and 0.34 ml acetic acid (Kemika, Croatia) and made up to 100.0 ml with water. Casein (Merck, Germany) as a precipitation agent was used. Folin–Ciocalteu's reagent (Merck, Germany) as a chromogenic agent was used. Filtration of prepared sample solutions was performed by using 0.20 μm Minisart-plus membrane filter (Sartorius AG, Germany).

2.3. Plant material

Wild growing *Plantago* species (*P. altissima* L., *P. argentea* Chaix, *P. coronopus* L., *P. holosteum* Scop. subsp. *holosteum*, *P. holosteum* subsp. *depauperata* Pilger, *P. holosteum* subsp. *scopulorum* (Degen) Horvatić, *P. lagopus* L., and *P. maritima* L.) were collected in June 2003 in Istria and on the islands of Cres and Lošinj, Croatia. 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 powdered samples of leaves, stems, and flowers were investigated.

2.4. Analytical procedures

2.4.1. Preparation of samples for prevalidation

Analyte stock standard solution was prepared by exact weighing of 10.0 mg tannin (Kemika, Croatia), dissolving in water and diluting to 100.0 ml with the same solvent. Secondary stock solution was made by mixing 5.0 ml of the standard solution and 5.0 ml of acetate buffer. In adequate volume of secondary stock solution (1.0, 0.8, 0.6, 0.4, 0.2, and 0.1 ml, corresponding to 50, 40, 30, 20, 10, and 5 μg of tannin, respectively), 0.5 ml of Folin–Ciocalteu's reagent was added. Each solution was made up in 10 ml volumetric flask with 33% Na_2CO_3, $10H_2O$. After filtration, the absorbance at 720 nm of the final blue solution was measured. Blank solution was prepared and measured identically, but without analyte.

2.4.2. Execution of prevalidation

Strategy of prevalidation measurements on standardized basis were based on a set of 24 blocks of data (six analytical groups of four experiments each) to relate measured values to blank values. Standards and blanks were measured in standard working range of one power of 10 ($x_1 = x_U = 50.0$ μg, upper level of analyte, $x_2 = 40.0$ μg, $x_3 = 30.0$ μg, $x_4 = 20.0$ μg, $x_5 = 10.0$ μg, $x_6 = x_L = 5.0$ μg, lower level of analyte), alternately in the following group sequence: 1, 6, 2, 5, 3, 4. Standardized mathematical/statistical procedure included descriptive and prognostic statistics. Descriptive statistics (arithmetic means, standard and relative standard deviations) was used for characterization of all analytical groups. Prognostic statistics included: checking of groups 1 and 6, testing of data homogeneity, establishment of calibration and analytical evaluation function, outlier recognition and estimation of limiting values. The prevalidation expert system and all algorithms used together with system of diagnosis were elaborated gradually in the paper [10].

2.4.3. Extraction and determination of polyphenols

The content of total polyphenols and tannins in *Plantago* species was determined by *FCR* procedure using Schneider's method [20]. Powdered plant material (0.25 g of each leaves, stems and flowers) was extracted with 80 ml of 30% methanol (70 °C, water bath, 15 min). After cooling and filtration, each extract was made up to 100.0 ml with 30% methanol (basic sample solution, BSS). Two milliliters of BSS was mixed with 8 ml of water and 10 ml of acetate buffer (solution 1, S1). Ten milliliters of S1 was shaken with 50 mg of casein during 45 min (adsorption of tannins) and then filtrated

(solution 2, S2). One milliliter of S1 was mixed with 0.5 ml of *FCR* and made up to 10.0 ml with 33% Na_2CO_3, $10H_2O$. The same procedure was performed with S2. After filtration, the absorbance at 720 nm of the final blue solution was measured. Blank solution was prepared and measured identically, but without analyte. The content of total polyphenols and tannins was evaluated upon three independent analyses. Absorbance values obtained for S1 correspond to total polyphenol content. Differences between absorbances of S1 and S2 correspond to concentration of casein-adsorbed tannins in plant samples. The content of total polyphenols and tannins was expressed as percentage toward the mass of dry herbal material.

2.4.4. Multivariate analysis

The results of polyphenols analysis were evaluated using multivariate analysis [21–23]. Cluster analysis was done with the unweighted pair-group method with arithmetic mean (UPGMA) using Euclidean distance (D_E). UPGMA generally yields results which are the most accurate for classification purposes [24–26]. Before the cluster analysis each variable was standardized [24,27–29]. 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 [27,30,31]. The statistical analysis of the results of polyphenols determination was performed using software Statistica 6.0.

3. Results and discussion

3.1. Analysis of prevalidation results

Starting data for prevalidation strategy [10] were mass of tannin (x) within the working range from 50.0 to 5.0 μg, absorbances obtained in measurements of the blank (B), the sample (y) and corrected absorbances (S). The extensive prevalidation metrological characteristics are summarized in Table 1.

3.1.1. Characterization of groups 1–6

Standard and relative standard deviation values showed that reasonable precision was attained for the absorbances obtained in measurements of the sample (s_{ry} from ±0.62 to ±3.48) and for corrected absorbances (s_{rS} from ±0.60 to ±3.43). Since lower level of precision was obtained for blank measurements (s_{rB} from ±5.82 to ±18.12), influence on the quality of results could be expected.

3.1.2. Checking of limiting groups 1 and 6

For the system under study blank signal is significantly lower then the gross signal at lower analyte level, significant influence of blank dispersions on s_M is not expected ($s_{rB1} = \pm 5.82\%$, $s_{rB6} = \pm 7.86\%$) and determination limit is expected below x_6. For the standard measurement, s_r values for both gross and corrected measurements at x_U and x_L lie below ±2.5% ($s_{ry1} = \pm 0.62\%$, $s_{rS1} = \pm 0.60\%$) and ±25% ($s_{ry6} = \pm 2.24\%$, $s_{rS6} = \pm 2.17\%$), respectively. Additional checking of quality of signal resolution for the *FCR* procedure showed that gross and blank signals were excellent distinguished ($R = 24.60$). The preliminary linearity check applied to A values (particular sensitivity values, $A = S/x$) for limiting groups 1 and 6 showed that linear calibration function is expected ($R = 3.05$).

3.1.3. Testing of data homogeneity

Analysis of variance applied to the six groups of blank values in *FCR* procedure indicated high homogeneity of blank values ($R = 0.92$). Additional checking of homogeneity showed that influence of blank values is not negligible

Table 1
Prevalidation characteristics of *FCR* procedure for tannin determination

Parameter						*FCR* procedure
Working range (μg)						50.0–5.0
Information value range (absorbance units)						0.34–0.04
Analyte–signal relationship						$r = 0.9981$
Calibration function						$\hat{S} = 0.0106x$
Analytical evaluation function						$\hat{x} = 94.15S$
Standard deviation of procedure						±1.03
Limit of detection, L_D (μg)						0.39
Limit of quantitation, L_Q (μg)						1.31
Groups data						
Actual (μg)	50.00	40.00	30.00	20.00	10.00	5.00
Found (μg)	50.01	41.05	29.01	19.36	10.54	5.04
Random deviations						
$s_{\hat{x}}$ (μg)	±0.30	±0.86	±0.91	±0.28	±0.34	±0.11
$s_{r\hat{x}}$ (%)	±0.60	±2.06	±3.14	±1.46	±3.43	±2.17
Systematic deviations, $\Delta\bar{x}$						
Absolute (μg)	−0.40	+1.60	−1.14	−0.85	+0.04	+0.14
Relative (%)	−0.81	+4.00	−3.80	−4.26	+0.38	+2.80

since they are not small enough in relation to information obtained at the upper analyte level. Moreover, total s_r value for blank measurements lie below ±50% ($s_{rBN} = \pm 13.37$). These results impose the need of correcting each y value with grand blank mean in *FCR* procedure. By using Barttlet test, analysis of six variances, applied to s and s_r values for B, y, S, A values, as well as to the values of the apparent mass of analyte ($\hat{x}$) pointed to strongly homogenous values of s_B, s_{rB}, s_{ry}, s_{rS}, s_A and s_{rA} as well as homogenous values of s_y and s_S.

3.1.4. Relation between signal and concentration

The characteristic data evaluated by method of the least squares were: determination coefficient ($r^2 = 0.99805$), slope of a line ($b = 0.0107$), intercept of a line ($a = -0.0029$), errors in the slope ($s_b = \pm 0.00067$) and intercept ($s_a = \pm 0.00106$). It was established that for the *FCR* procedure significant correlation does exist ($R = 75.09$). Furthermore, complete and deep evaluation of calibration function [10] showed that both ideal calibration and analytical evaluation function were found (Table 1). Other characteristic data were mean errors of calibration ($s_v = \pm 0.00007$) and analytical evaluation constants ($s_v = \pm 0.6548$) and the standard deviation of the analytical procedure ($s_M = \pm 1.0333$) in the given working range. 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.

3.1.5. Outlier recognition

Since one outlying value observed in *FCR* procedure is in accordance with prevalidation acceptance criteria, there is no objection on the homogeneity of the data material.

3.1.6. Estimation of limiting values

According to up-to-date recommendations [6,32,33], limit of detection ($L_D = 0.39\ \mu g$) and limit of quantitation ($L_Q = 1.31\ \mu g$) were significantly lower than the mass of analyte at lower analyte level, x_6.

Analysis of variance, the Barttlet test, reality of linear analytical evaluation function, agreement of actual, x, and appropriate, $\hat{x}$ values as well as random and systematic deviations gave information on good quality of the analytical procedure.

3.2. Quantitative analysis of total polyphenols and tannins in Plantago L. species

Results of quantitative analysis of total polyphenols in leaves, stems, and flowers of *Plantago* species are given in Table 2.

The results of spectrophotometric determination of total polyphenols in examined plant species showed that generally the highest polyphenols content was observed in leaves (up to 10.15%, *P. holosteum* subsp. *holosteum*). The maximum concentration in stems was 4.34% (*P. holosteum* subsp. *scopulorum*), while the sample of *P. argentea* contained the greatest amount of total polyphenols in flowers (5.56%).

Table 2
Content of total polyphenols in different plant organs of *Plantago* L. species

Plant	Total polyphenols (%); $\bar{X} \pm$ S.D., $n = 3$		
	Leaves	Stems	Flowers
P. altissima	4.55 ± 0.16	3.57 ± 0.10	3.07 ± 0.09
P. argentea	7.51 ± 0.30	3.68 ± 0.21	5.56 ± 0.18
P. coronopus	5.45 ± 0.25	3.55 ± 0.17	3.13 ± 0.11
P. holosteum subsp. *depauperata*	5.05 ± 0.21	3.25 ± 0.13	2.79 ± 0.09
P. holosteum subsp. *holosteum*	10.15 ± 0.52	4.13 ± 0.29	3.91 ± 0.22
P. holosteum subsp. *scopulorum*	7.39 ± 0.31	4.34 ± 0.25	4.93 ± 0.07
P. lagopus	4.59 ± 0.26	3.15 ± 0.16	3.71 ± 0.15
P. maritima	7.32 ± 0.35	2.80 ± 0.23	3.57 ± 0.08

The contents of casein-adsorbed tannins are presented in Table 3. The samples of stems contained smaller quantities of tannins (0.28–1.00%) in comparison with samples of leaves and flowers, where maximum tannin concentrations of 2.26% (*P. argentea*) and 2.21% (*P. holosteum* subsp. *scopulorum*) were determined, respectively.

3.3. Mathematical/statistical evaluation of polyphenol analysis in Plantago L. species

According to content of total polyphenols, UPGMA separated investigated *Plantago* species in two main groups (Fig. 1). One cluster is constituted of three species: *P. argentea*, *P. holosteum* subsp. *scopulorum* and *P. holosteum* subsp. *holosteum* ($D_E = 2.09$). At the Euclidean distance of 3.03, this cluster was connected to the group of remained species, where the most similar species were *P. altissima* and *P. coronopus* ($D_E = 0.47$). Similar results to cluster analysis of total polyphenols were obtained by PCA, which separated group of three species (*P. holosteum* subsp. *holosteum*, *P. argentea*, and *P. holosteum* subsp. *scopulorum*) with the highest content of these compounds.

Table 3
Content of tannins in different plant organs of *Plantago* L. species

Plant	Tannins (%); $\bar{X} \pm$ S.D., $n = 3$		
	Leaves	Stems	Flowers
P. altissima	1.07 ± 0.15	0.28 ± 0.07	0.44 ± 0.09
P. argentea	2.26 ± 0.20	0.81 ± 0.16	0.96 ± 0.18
P. coronopus	0.82 ± 0.12	1.00 ± 0.12	1.07 ± 0.11
P. holosteum subsp. *depauperata*	1.32 ± 0.21	0.50 ± 0.08	0.72 ± 0.09
P. holosteum subsp. *holosteum*	1.99 ± 0.30	0.87 ± 0.21	1.03 ± 0.22
P. holosteum subsp. *scopulorum*	0.86 ± 0.05	0.56 ± 0.05	2.21 ± 0.07
P. lagopus	0.56 ± 0.17	0.84 ± 0.13	1.36 ± 0.15
P. maritima	0.93 ± 0.13	0.49 ± 0.09	0.72 ± 0.08

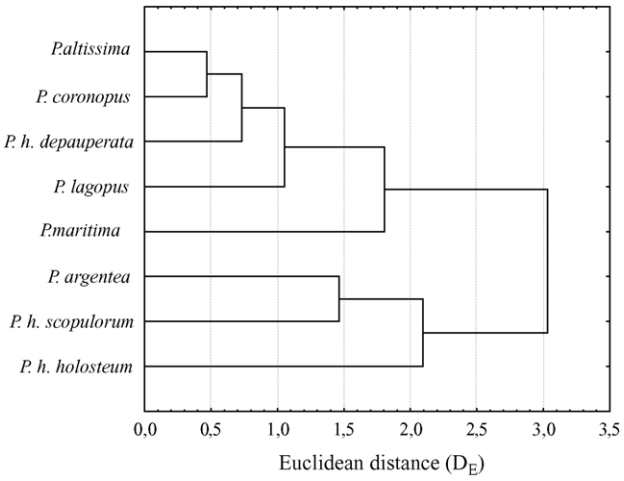

Fig. 1. UPGMA dendrogram of total polyphenols in *Plantago* L. species.

Quite different results were obtained as regard to content of tannins as a group of polyphenolic compounds. Toward tannins, examined *Plantago* species were separated in two main clusters, which were connected at linkage distance of 2.70 (Fig. 2). The greatest similarity was observed between *P. argentea* and *P. holosteum* subsp. *holosteum* ($D_E = 0.53$). According to tannins, it was established that PCA divided herbal species in two groups: *P. argentea* and *P. holosteum* subsp. *holosteum* (with the greatest amount of tannins) were separated from the others.

As regard to both contents of total polyphenols and tannins in leaves, stems and flowers, two main clusters were obtained by UPGMA. One cluster formed species *P. argentea* and *P. holosteum* subsp. *holosteum* ($D_E = 2.42$), and *P. holosteum* subsp. *scopulorum*, which was connected to them at $D_E = 3.69$. The rest of examined species formed the other cluster. The most similar were *P. altissima* and *P. holosteum* subsp. *depauperata* ($D_E = 1.34$), as well as *P. coronopus* and *P. lagopus* ($D_E = 1.44$). Two main clusters were connected at

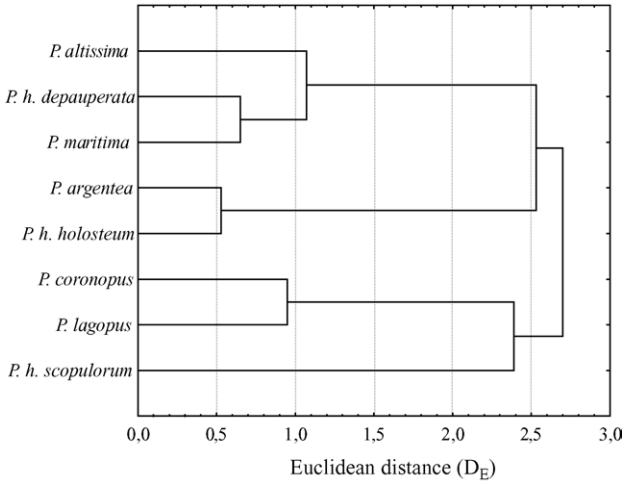

Fig. 2. UPGMA dendrogram of tannins in *Plantago* L. species.

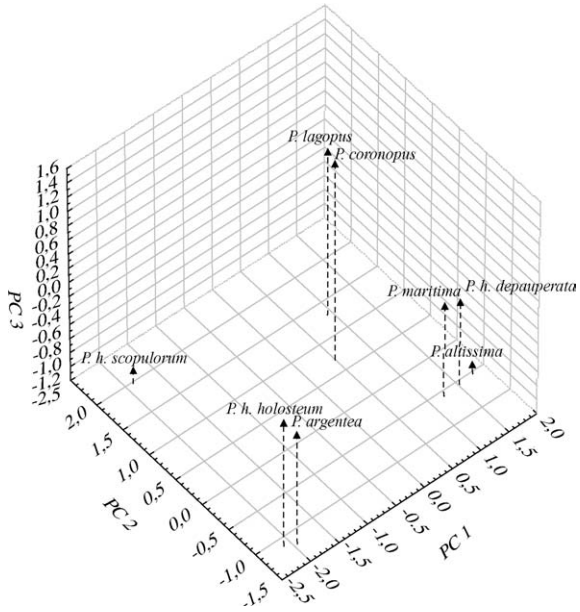

Fig. 3. PCA of total polyphenols and tannins in *Plantago* L. species.

Table 4
Eigen-vectors of the principal components

Variable	PC 1	PC 2	PC 3
Total polyphenols (leaves)	−0.473885	−0.284583	−0.008198
Total polyphenols (stems)	−0.465655	0.169282	−0.316347
Total polyphenols (flowers)	−0.488787	0.055494	−0.149965
Tannins (leaves)	−0.356995	−0.646629	−0.019577
Tannins (stems)	−0.275014	0.103857	0.933549
Tannins (flowers)	−0.341478	0.677024	−0.073961

the Euclidean distance of 4.02. PCA gave the similar results as corresponding cluster analysis in the case of both contents of total polyphenols and tannins (Fig. 3). The first principal component explains 47.27% of the total variance, the second one 23.09% and the third component explains 14.50% of the variance. Accordingly, the first three components account for 84.85% of the variance, which points to valuable results of PCA.

Eigen-vectors matrix with the loading of each variable in each principal component is presented in Table 4. The highest contribution to the first PC axis gave the total polyphenols (first in flowers, then in leaves and finally in stems). Furthermore, the content of tannins in flowers and leaves contributed the most to the second PC axis, while the maximum score for PC 3 was obtained from the content of tannins in stems.

4. Conclusion

Prevalidation strategy was used to obtain prevalidation characteristics of spectrophotometric procedure for determination of polyphenols with Folin–Ciocalteu's reagent. Favourable metrological characteristics 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 = 1.31 μg) and favourably random (from ±0.60% to ±3.43%) and systematic (from −4.26% to +4.00%) deviations accordant with prevalidation acceptance criteria.

The results of polyphenol analysis in *Plantago* species performed by *FCR* procedure showed that leaves generally contained the greater amount of total polyphenols and tannins compared to stems and flowers of investigated plants. The highest concentration of total polyphenols was determined in leaves of *P. holosteum* subsp. *holosteum* (10.15%), while the highest content of tannins was obtained in leaves of *P. argentea* (2.26%) and they could be considered as a good resource of these biologically active compounds.

Multivariate analysis (UPGMA and PCA) of total polyphenols and tannins in *Plantago* species pointed out two species (*P. argentea* and *P. holosteum* subsp. *holosteum*) with the highest amount of these compounds. In the case of UPGMA analysis of total polyphenols, the most similar species were *P. altissima* and *P. coronopus* (D_E = 0.47), while according to cluster analysis of tannins, the most similar species were *P. argentea* and *P. holosteum* subsp. *holosteum* (D_E = 0.53).

Ultimately, the present study showed that the prevalidation strategy has proven valuable for evaluating the validity of *FCR* procedure, which was successfully applied for polyphenols determination in plant material. Moreover, the obtained results of performed phytochemical and multivariate analysis have contributed to the chemotaxonomical research of genus *Plantago*.

References

[1] Y. Vander Heyden, F. Questier, D.L. Massart, J. Pharm. Biomed. Anal. 17 (1998) 153–168.
[2] Y. Vander Heyden, F. Questier, L. Massart, J. Pharm. Biomed. Anal. 18 (1998) 43–56.
[3] Y. Vander Heyden, S. Kuttathharmmakul, J. Smeyers-Verbeke, D.L. Massart, Anal. Chem. 72 (2000) 2869–2874.
[4] CPMP/ICH Working Party on Quality of Medicinal Products, Analytical Validation, Commission of the European Communities, 1989, pp. 1–9.
[5] ICH Harmonized Tripartite Guideline prepared within the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) Q2A, Text on Validation of Analytical Procedures, 1994 (available from http://www.ich.org/ich5q.html).
[6] ICH Harmonized Tripartite Guideline prepared within the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) Q2B, Validation of Analytical Procedures: Methodology, 1996 (available from http://www.ich.org/ich5q.html).
[7] Council of Europe, European Department for the Quality of Medicines, Technical Guide for the Elaboration of Monographs, second ed., Pharmeuropa, 1996, pp. 1–42 (special issue).
[8] U.S. Food and Drug Administration, Draft Guidelines on the Validation of Analytical Procedures: Methodology, Federal Register 61 (1996) 9316–9319.
[9] The United States Pharmacopeia, Section 1225, Validation of Compendial methods, United States Pharmacopeial Convention, Rockville, 2002, pp. 2256–2259.
[10] V. Grdinić, J. Vuković, J. Pharm. Biomed. Anal. 35 (2004) 489–512.
[11] C.A. Rice-Evans, N.J. Miller, G. Paganga, Trends Plant Sci. 2 (1997) 152–159.
[12] M. Filipiak, Anal. Sci. 17 (2001) i1667–i1670.
[13] A. Brantner, Ž. Maleš, S. Pepeljnjak, A. Antolić, J. Ethnopharmacol. 52 (1996) 119–122.
[14] R. Taskova, Lj. Evstatieva, N. Handjieva, S. Popov, Z. Naturforsch. 57c (2002) 42–50.
[15] A.B. Samuelsen, J. Ethnopharmacol. 71 (2000) 1–21.
[16] S.R. Jensen, C.E. Olsen, K. Rahn, J.H. Rasmussen, Phytochemistry 42 (1996) 1633–1636.
[17] S.A. Kawashty, E. Gamaleldin, M.F. Abdalla, N.A.M. Saleh, Biochem. Syst. Ecol. 222 (1994) 729–733.
[18] R. Jurišić, Ž. Debeljak, S. Vladimir-Knežević, J. Vuković, Z. Naturforsch. 59c (2004) 27–31.
[19] M. Paz Velázquez Fiz, A.M. Díaz Lanza, L. Fernández Matellano, Z. Naturforsch. 55c (2000) 877–880.
[20] G. Schneider, Arch. Pharm. 309 (1976) 38–44.
[21] B.G.M. Vandeginste, D.L. Massart, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics: Part B, Elsevier Science, Amsterdam, 1998, pp. 349–380.
[22] K. McGarigal, S. Cushman, S. Stafford, Multivariate Statistics for Wildlife and Ecology Research, Springer-Verlag, New York, 2000.
[23] S. Sharma, Applied Multivariate Techniques, John Wiley & Sons Inc., 1996.
[24] P.H.A. Sneath, R.R. Sokal, Numerical Taxonomy: The Principles and Practice of Numerical Classification, W.H. Freeman and Co., San Francisco, 1973, p. 201–209.
[25] R.R. Sokal, Annu. Rev. Ecol. Sys. 17 (1986) 423–442.
[26] F.J. Rohlf, M.C. Wooten, Evolution 42 (1988) 581–595.
[27] M. Höft, S.K. Barik, A.M. Lykke, Quantitative Ethnobotany: Applications of Multivariate and Statistical Analyses in Ethnobotany, People and Plants Working Paper 6, UNESCO, Paris, 1999, p. 9–21.
[28] R.R. Sokal, Syst. Zool. 10 (1961) 70–79.
[29] C.D. Michener, R.R. Sokal, Evolution 11 (1957) 130–162.
[30] J.H. Mook, J. Haeck, J. van der Toorn, P.H. van Tienderen, Ecology of *Plantago* populations, in: P.J.C. Kuiper, M. Bos (Eds.), *Plantago*: A Multidisciplinary Study, Springer-Verlag, Berlin, 1992, pp. 69–87.
[31] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, Pearson Education Limited, Essex, 2000.
[32] IUPAC, Harmonized guidelines for single-laboratory validation of methods of analysis, Pure Appl. Chem. 74 (2002) 835–855.
[33] D.L. Massart, B.G.M. Vanderginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics: Part A, Elsevier, Amsterdam, 1997.