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Application of conventional UV, photodiode array (PDA) and fluorescence (FL) detection to analysis of phenolic acids in plant material and pharmaceutical preparations

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Abstract

Free phenolic acids (PhAs) contained in methanolic extracts of *Eleutherococcus senticosus* roots and pharmaceutical preparations, deriving from this plant, were isolated by solid-phase extraction (SPE) and identified by reversed-phase high-performance liquid chromatography (RP-HPLC). To obtain precise, accurate and validated results of qualitative and quantitative analysis, ultraviolet (at $\lambda = 254$ nm), photodiode array (at $\lambda = 254$ and 280 nm) and fluorescence (at $\lambda_{Ex} = 230$ or 265 nm and $\lambda_{Em} = 350$ nm) detection was used. Additionally, the HPLC separation of PhAs on two different octadecyl sorbents: Hypersil™ (Shandon, UK) and Symmetry™ (Waters, USA) was performed. Eight PhAs: chlorogenic, protocatechuic, *p*-hydroxybenzoic, caffeic, vanillic, syringic, *p*-coumaric and ferulic, in different quantitative proportions, were identified, both in the roots of *E. senticosus* and pharmaceutical formulations examined.

Keywords: *Eleutherococcus senticosus*; Phenolic acids; Solid-phase extraction; High-performance liquid chromatography; UV–vis, PDA and FL detection

1. Introduction

Eleutherococcus senticosus (Rupr. et Maxim.) Maxim. = *Acanthopanax senticosus* (Rupr. et Maxim.) Harms, belonging to the Araliaceae family, is a shrub indigenous to the northern regions of Russia (Siberia) and the northeastern parts of China and Korea. This plant became very popular especially in the last three decades because of the therapeutic properties of its roots. They are considered to have a similar pharmacological action to Chinese ginseng. The biologically active constituents, showing adaptogenic activity, are heterogeneous compounds known as eleuterosides. They comprise phenylpropanoids, lignans, sterols, coumarins, and mono- and polysaccharides (glucans) [1,2]. In recent years more and more clinical studies were focused on phenolic acids (PhAs) as a group of potentially immunostimulating com-

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pounds, i.e. plant inducers of fagocytosis and the synthesis of interferons and antibodies in humans [3–6]. Additionally, PhAs possess antiviral, antibacterial, anti-inflamatory and antioxidant properties [7]. That is why we dealt with qualification and quantification of free PhAs in *E. senticosus* and respective phytopharmaceuticals.

Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with UV spectrometry has been used predominantly as an analytical method for a wide range of PhAs in plant material [8–14]. As the separation of these compounds in complex plant extracts yields some problems (e.g. there are slight differences in retention times among several components), in this study a classical ultraviolet (UV), together with a photodiode array (PDA) and a fluorescence (FL) detector were used for monitoring of PhAs in samples examined. Additionally, two different (Hypersil and Symmetry) 5-μm reversed-phase stationary phases were used. To obtain highly purified fractions of free PhAs, a selective and rapid SPE procedure was performed.

2. Materials and methods

2.1. Chemicals

Methanol, acetic acid, phosphoric acid, of HPLC-grade, were obtained from J.T. Baker, (Deventer, Holland). Sodium bicarbonate, sodium hydroxide, hydrochloric acid, diethyl ether (of analytical grade) were provided by Merck (Darmstadt, Germany) and petroleum ether by POCh (Gliwice, Poland). In all experiments bidistilled water was used. Standards of PhAs were purchased from Sigma (St. Louis, MO, USA).

2.2. Plant material and pharmaceutical preparations

The roots of *E. senticosus* were obtained from the China National Pharmaceutical Foreign Trade Corporation (Beijing, China). Commercially available pharmaceutical preparations: Extractum *Eleutherococci fluidum* (Łubnyfarm, Łubny, Ukrain), Eleuterosol (Herbapol-Lublin, Lublin, Poland.) and Siberian Ginseng (Lifeplan Products, Lutterworth, UK), containing the extracts from the roots of *E. senticosus*, were examined.

2.3. Sample preparation

Dry, pulverized samples (2 g) of *E. senticosus* roots (or micronized tablets of the pharmaceutical preparation Siberian Ginseng) were refluxed with methanol (50 ml) on a water bath for 1 h. Liquid was carefully decanted and the plant material was reextracted with the same solvent (2 × 50 ml). All supernatants were combined, partially evaporated under reduced pressure, at 50°C, filtered and placed in 25-ml volumetric flasks.

2.4. SPE procedure

Isolation of free PhAs fractions from plant material and pharmaceutical preparations was carried out according to our own SPE procedure, elaborated and first used for the selective separation of these compounds from some Echinacea species [15].

Samples (5 ml) of methanolic extracts from the roots of *E. senticosus* and the tablets of Siberian Ginseng or ethanolic extracts from *E. senticosus*, corresponding with pharmaceutical preparations: Eleuterosol and Extractum *Eleutherococci fluidum*, were evaporated to dryness, diluted with 30% aqueous methanol and passed under vacuum through preliminary conditioned (with 10 ml methanol, followed by 10 ml bidistilled water) octadecyl BakerBond SPE-cartdridges (500 mg, 3 ml; J.T. Baker, Phillipsburg, NJ, USA). For the SPE procedure a vacuum manifold processor (system SPE-12G, J.T. Baker, Großgerau, Germany) was used. Eluates, purified from the ballast compounds and containing the complex of phenolics, were further adjusted to pH 7.0–7.2 with 5% sodium bicarbonate aqueous solution and passed (under reduced pressure — 0.01 MPa) through quaternary amine BakerBond SPE-cartridges (500 mg, 3 ml; J.T. Baker). They were conditioned immediately prior to use with bidistilled water (10 ml) followed by 0.15% sodium bicarbonate aqueous solution (20 ml). After passing the sam-

ples through the sorbent beds, the cartridges were dried under vacuum and the analytes (PhAs) were desorbed and rinsed off into collection vials using 0.2 M phosphoric acid and methanol (1:1 v/v) — 5 ml for each sample. The eluates were adjusted to pH 3with 1 M sodium hydroxide and subsequently qualitatively and quantitatively analysed by RP-HPLC.

2.5. Classical LLE procedure

Methanolic extracts of the roots of *E. senticosus* (see Section 2.3) concentrated in vacuo, were diluted with hot, distilled water (50 ml), and filtered. Water extracts were partitioned into several fractions, according to a liquid–liquid extraction (LLE) procedure elaborated for PhAs [16]. For this purpose, each of the water extracts was purified by shaking with petroleum ether (2×20 ml) — the organic layer was rejected after extraction — then extracted (10×20 ml) with diethyl ether (Et_2O), finally providing water and Et_2O layers. PhAs contained in the Et_2O layers were further treated with 5% sodium bicarbonate ($10 \times$ 10 ml). Bicarbonate layers were acidified with 36% hydrochloric acid until pH 3 and, thereafter, extracted with Et_2O (10×20 ml). Each of Et_2O extracts was evaporated to dryness, and the residues were dissolved in methanol (10 ml), giving fractions of free PhAs.

2.6. HPLC analysis

Two separate chromatographic systems (in two independent laboratories) were used for HPLC analysis of PhAs.

The first one consisted of a Hewlett-Packard (Palo Alto, CA, USA) model 1050 liquid chromatograph equipped with a Rheodyne injector with a 20-μl sample loop and a variable wavelength UV–vis detector set at 254 nm. A stainless-steel column (200×4.6 mm ID) packed with 5-μm ODS Hypersil (Shandon, Cheshire, UK) was used for the separation of PhAs with a mobile phase: methanol/water/acetic acid (23:77:1, v/v/v), at a flow-rate of 1 ml/min and ambient temperature. The chromatograms were recorded using a HP 3396 series II integrator (Palo Alto, CA, USA).

The second chromatographic system comprised a Hewlett-Packard model 1100 HPLC system (Waldbronn, Germany) equipped with a quaternary pump, a 1100 series PDA and a 1100 series FL detectors, a vacuum degasser, a thermostatted column compartment, and an autosampler. For spectral acquisition and evaluation a Hewlett-Packard 3D ChemStation (Waldbronn, Germany) was used. Separations were performed at 30°C, on a 5-μm Symmetry™ C_{18} column (250×4.6 mm ID; Waters, Milford, MA, USA) with methanol/0.001 M phosphoric acid (23:77 v/v). Chromatography was carried out under isocratic conditions, at a flow-rate of 1 ml/min.

The identity of the separated peaks was assigned by co-chromatography with the authentic standards. Quantification of PhAs was carried out by integration of the peak areas using an external standard method. Calibration curves in the range 10–100 μg/ml have been obtained by injecting separately standard solutions with a concentration of 10, 25, 50, 75 and 100 μg/ml of each PhA. They show a linear relationship between the instrumental response and the analytes concentration with correlation coefficient ranging from 0.9991 to 0.9996 for compounds examined.

3. Results and discussion

3.1. Optimisation of detection parameters

A PDA detector, used in our study, enabled scanning of the UV spectrum of the solutes passing the detector cell and this, together with the retention time (t_R) values of the peaks examined, was of great importance for purposes of identification of PhAs. Systematic studies on the absorbance spectra of PhAs standards carried out on a PDA detector allowed to establish two maxima: at 254 and 280 nm which were common for the majority of compounds examined. Our results were also in agreement with those obtained in other studies [8–14]. The chromatogram of the sample examined (Eleuterosol), containing the isolated fraction of free PhAs, monitored at 280 nm using a PDA detector is presented in Fig. 1. More difficult problem in our experiment was to

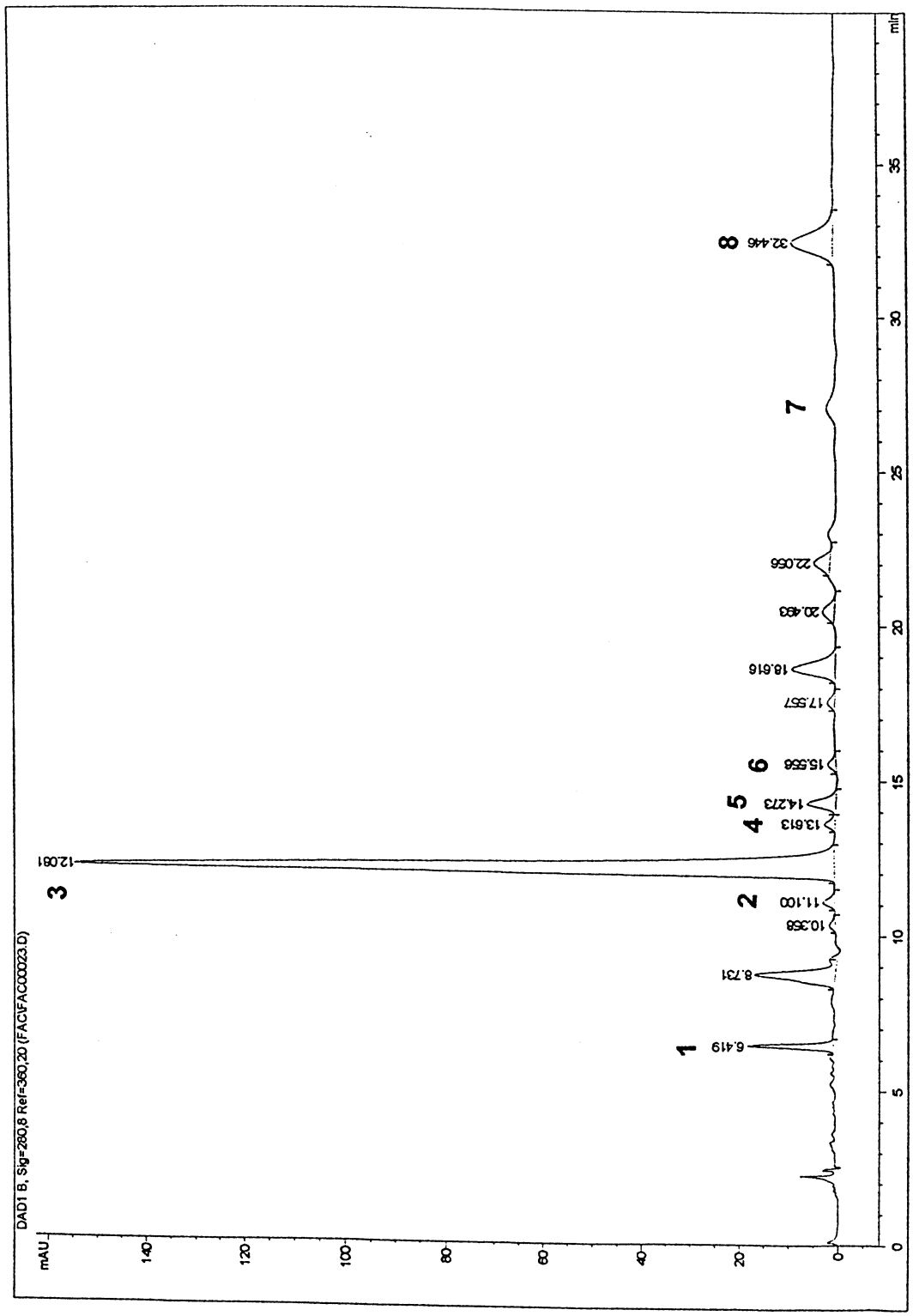

Fig. 1. HPLC separation of PhAs from the pharmaceutical preparation Eleuterosol using the Symmetry RP-18 column. Mobile phase: methanol/0.001 M phosphoric acid (23:77 v/v); flow-rate: 1.0 ml/min; column temperature: 30°C; detection: PDA at 280 nm. Peaks of PhAs: 1, protocatechuic; 2, chlorogenic; 3, *p*-hydroxybenzoic; 4, vanillic; 5, caffeic; 6, syringic; 7, *p*-coumaric; 8, ferulic.

establish the correct excitation and emission wavelengths for a FL detector, as high differences in the absorbance and fluorescence intensity for several PhAs were observed. Fortunately, the modern 1100 rapid scanning FL detector used allowed for either the application of multiple excitation and emission wavelengths at any given time, and acquisition of spectral information in the continuous flow mode. Finally, as optimal, two excitation wavelengths: at 230 and 265 nm and the emission wavelength at 350 nm were chosen for the qualitation of PhAs. A representative chromatogram is shown in Fig. 2. For the determination of PhAs in samples examined the excitation wavelength $\lambda = 265$ nm was used.

3.2. Optimisation of chromatographic conditions

The composition of the mobile phase was selected in such a way that all PhAs were resolved in the shortest analysis time possible. Having considered the different parameters, the optimum conditions for the separation of PhAs, using both the Hypersil RP-18 and the Symmetry RP-18 columns, were 23% of methanol in acidified (with acetic or phosphoric acid) to pH 3–3.5 water as a mobile phase. As the reversed-phase separation of PhAs often results in broad, tailing bands, which are caused by acidic sites (residual free silanols) present in the column packing, the use of acidic components in the mobile phase minimised this problem by the producing of 'ion-suppression' effect.

3.3. Accuracy and precision

In earlier investigations [15], in order to establish the efficiency of the applied SPE method, recovery tests were also performed for the PhAs standards. Methanolic (30%) solutions (10 ml) of 1 mg caffeic and *p*-hydroxybenzoic acids (as the representatives of the derivatives of cinnamic and benzoic acids) were submitted to the SPE procedure. A percentage recovery of $98.5 \pm 0.5\%$ ($n = 5$) for these compounds was obtained.

In this study, the accuracy of the SPE method was determined by comparing the results obtained by the proposed SPE technique with a traditional LLE procedure (Table 1). Based on these results, SPE procedure seems to be a very effective technique for separation of PhAs from plant extracts, especially for some cinnamic acid derivatives (chlorogenic, ferulic, caffeic).

In our investigations, limits of detection (LOD) and quantification (LOQ) for PhAs, using the PDA detector at the operative wavelength of 280 nm and the FL detector at $\lambda_{Ex} = 265$ nm, were also determined (Table 2).

The method precision was calculated by SPE using adequate solutions and sorbents and HPLC assay of independent samples ($n = 5$) from the same material. On the basis of quantitative results and their statistical evaluation in the Table 3 presented, the SPE-HPLC combined with variable detection should be described as the repeatable and precise method of quantification of PhAs. The small differences in the quantitative results

Table 1
Levels of PhAs (in μg/g dry wt) extracted from the roots of *E. senticosus* using SPE compared with conventional LLE

No	PhAs (names)	LLE[a]	SPE[a]	Relative recovery[b] (LLE/SPE)
1	Protocatechuic	70.05 (3.5)	71.83 (4.7)	97.5
2	Chlorogenic	99.96 (10.6)	3502.51 (5.1)	2.9
3	*p*-Hydroxybenzoic	0.65 (15.3)	1.24 (10.4)	52.4
4	Vanillic	5.19 (12.1)	7.65 (10.6)	67.8
5	Caffeic	35.87 (11.9)	100.46 (3.9)	35.7
6	Syringic	23.44 (11.1)	24.39 (3.9)	96.1
7	*p*-Coumaric	8.82 (15.7)	16.67 (8.6)	52.9
8	Ferulic	4.37 (8.9)	179.17 (4.0)	2.4

[a] Each value is the mean (R.S.D.) of five determinations.
[b] Percentage recovery based on the amount extracted by the SPE method.

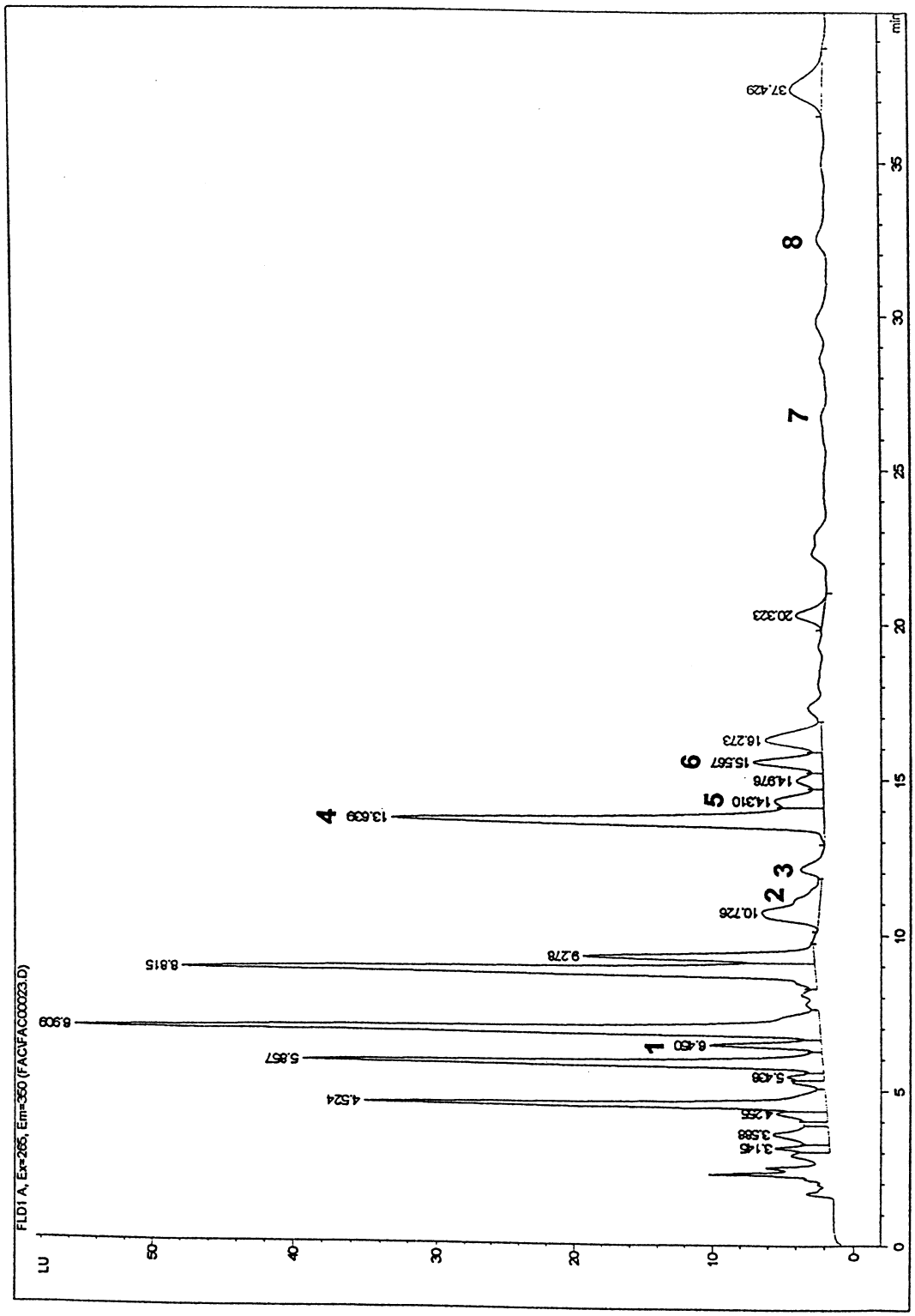

Fig. 2. Elution profile by RP-HPLC of PhAs contained in the pharmaceutical preparation Eleuterosol analysed fluorimetrically using excitation and emission wavelengths at 265 and 350 nm, respectively. Other chromatographic conditions and peaks of PhAs as in Fig. 1.

Table 2
Limit values for PhAs, determined using PDA ($\lambda = 280$ nm) and FL ($\lambda_{Ex} = 265$ nm) detectors (PhAs numbered as in Table 1)

Comp. No	PDA (ng/ml)	FL (ng/ml)
1	93[a]	96[a]
	282[b]	291[b]
2	227[a]	4167[a]
	757[b]	12627[b]
3	33[a]	11[a]
	100[b]	33[b]
4	50[a]	3[a]
	151[b]	9[b]
5	73[a]	1463[a]
	221[b]	4433[b]
6	40[a]	9[a]
	121[b]	27[b]
7	44[a]	1250[a]
	133[b]	3788[b]
8	107[a]	1429[a]
	324[b]	4330[b]

[a] LOD.
[b] LOQ.

using UV and PDA detectors can be explained by a different mode of data acquisition and integration in Hewlett-Packard model 1050 and 1100 chromatographic sytems and probable slight differences in operation procedures used in the two laboratories.

3.4. Results of qualitative and quantitative analysis

The practical purpose of this study was to determine the real levels of free PhAs in the roots of *E. senticosus* and pharmaceutical preparations. Using RP-HPLC, the following representatives of PhAs were identified: protocatechuic, *p*-hydroxybenzoic, chlorogenic, caffeic, vanillic, syringic, *p*-coumaric and ferulic. Quantities of the individual compounds varied significantly. Chlorogenic acid turned out to be the most predominant PhA in the roots of *E. senticosus* (the mean content above 3500 μg/g dry wt) and a pharmaceutical preparation Eleuterosol (about 1800 μg/ml), deriving from the same Chinese plant material (Table 3). The next two high concentrations for ferulic and caffeic acids were established in the above samples

Table 3
The content of PhAs in the roots of *E. senticosus* and pharmaceutical preparations: Eleuterosol, Extractum *Eleutherococci fluidum*, Siberian Ginseng (the results obtained using (A) the UV detector and the Hypersil RP-18 (5 μm) column, (B) the PDA detector and the Symmetry RP-18 (5 μm) column, (C) the FL detector and the Symmetry RP-18 (5 μm) column, PhAs numbered as in Table 1)

Comp. No	A	B	C
1	71.83 (4.7)[a]	81.11 (1.3)[a]	76.674 (6.1)[a]
	24.07 (10.1)[b]	31.54 (3.5)[b]	30.02 (3.3)[b]
	50.89 (8.1)[c]	60.02 (2.0)[c]	59.63 (3.4)[c]
	32.79 (8.4)[d]	34.23 (6.3)[d]	35.87 (4.2)[d]
2	3502.51 (5.1)[a]	3077.01 (4.0)[a]	3424.24 (3.8)[a]
	1933.53 (2.3)[b]	1739.73 (4.0)[b]	1805.52 (3.7)[b]
	227.68 (13.6)[c]	200.50 (3.8)[c]	252.26 (2.6)[c]
	105.06 (11.5)[d]	95.96 (5.8)[d]	91.23 (3.3)[d]
3	1.24 (10.4)[a]	1.44 (8.3)[a]	1.88 (12.2)[a]
	1.09 (8.3)[b]	1.13 (10.6)[b]	1.26 (8.7)[b]
	2.95 (11.5)[c]	2.80 (2.8)[c]	3.03 (8.3)[c]
	4.17 (9.5)[d]	4.95 (8.2)[d]	4.54 (6.3)[d]
4	7.65 (10.6)[a]	5.88 (2.2)[a]	5.41 (12.1)[a]
	7.00 (10.8)[b]	4.89 (3.7)[b]	5.90 (2.3)[b]
	8.30 (9.0)[c]	6.43 (4.7)[c]	6.75 (2.8)[c]
	12.45 (6.7)[d]	10.54 (6.9)[d]	10.98 (7.2)[d]
5	100.46 (3.9)[a]	94.69 (4.8)[a]	117.85 (8.0)[a]
	33.50 (11.6)[b]	30.32 (7.4)[b]	36.86 (6.7)[b]
	310.28 (5.6)[c]	292.79 (2.3)[c]	349.88 (2.9)[c]
	37.13 (4.6)[d]	39.84 (3.2)[d]	38.56 (2.4)[d]
6	24.39 (3.9)[a]	16.87 (2.3)[a]	17.85 (6.8)[a]
	6.94 (12.9)[b]	6.53 (13.7)[b]	7.07 (8.8)[b]
	51.38 (12.4)[c]	41.06 (8.6)[c]	45.98 (6.9)[c]
	16.71 (10.7)[d]	14.9 (2.3)[d]	15.01 (5.8)[d]
7	16.67 (8.6)[a]	15.08 (2.4)[a]	19.95 (10.9)[a]
	5.78 (9.1)[b]	7.43 (9.6)[b]	8.15 (2.3)[b]
	14.48 (7.8)[c]	11.39 (4.1)[c]	13.77 (6.8)[c]
	42.07 (10.0)[d]	44.21 (5.5)[d]	44.02 (2.7)[d]

Table 3 (*Continued*)

Comp. No	A	B	C
8	179.17 (4.0)[a]	159.84 (4.3)[a]	173.72 (9.8)[a]
	76.99 (10.9)[b]	77.03 (10.3)[b]	81.59 (6.1)[b]
	18.33 (9.2)[c]	18.18 (6.6)[c]	23.94 (9.0)[c]
	30.99 (5.1)[d]	31.78 (2.9)[d]	30.02 (4.5)[d]

[a] *E. senticosus*, μg/g dry weight; mean (R.S.D.); $n = 5$.
[b] Eleuterosol, μg/ml; mean (R.S.D.); $n = 5$.
[c] Extractum *Eleutherococci fluidum*, μg/ml; mean (R.S.D.); $n = 5$.
[d] Siberian Ginseng, μg/tablet; mean (R.S.D.); $n = 5$.

examined. In opposition to these results, the preparation Extractum *Eleutherococci fluidum*, produced on the basis of the plant material deriving from Ukraine, contained considerably less amounts of PhAs and the highest concentration (approximately 300 μg/ml) for caffeic acid was determined (Table 3). The most comparable amounts (the least differentiation among several compounds) of all PhAs in the tablets of Siberian Ginseng are stated in Table 3. However, similarily to Eleuterosol, the highest concentration for chlorogenic acid was documented (Table 3).

The presence of high amounts of cinnamic acid derivatives (chlorogenic, caffeic) possessing immunostimulating properties suggest that these components may be another interesting group of pharmacologically active compounds contained in the roots of *E. senticosus* and respective pharmaceutical formulations.

4. Conclusions

The application of the effective SPE procedure in combination with variable (UV, PDA, FL) detection made the proposed SPE-HPLC method sensitive, reproducible and superior to traditional techniques used for the determination of PhAs in plant material and pharmaceutical preparations.

References

[1] C.A. Newall, L.A. Anderson, J.D. Phillipson, Herbal Medicines. A Guide for Health-Care Professionals, The Pharmaceutical Press, London, 1996.
[2] J. Fang, A. Proksch, H. Wagner, Phytochemistry 24 (1985) 2619–2622.
[3] E. Skopińska-Różewska, H. Rządkowska-Bodalska, W. Olechnowicz-Stępień, et al., Bull. Pol. Acad. Sci. 39 (1991) 257–266.
[4] T. Sawicka, J. Drozd, J. Prosińska, B. Borkowski, Herba Pol. 40 (1994) 31–34.
[5] T. Skwarek, Z. Tynecka, K. Głowniak, E. Lutostańska, Herba Pol. 40 (1994) 42–49.
[6] J. Drozd, Biul. Inst. Lek. 39 (1995) 13–24.
[7] J.L. Lamaison, C. Petitjean-Freytet, F. Duband, A.P. Carnat, Fitoterapia 52 (1991) 166–167.
[8] S. Caccamese, R. Azzolina, Chromatographia 12 (1979) 545–547.
[9] J.M. Andersen, W.B. Pedersen, J. Chromatogr. 259 (1983) 131–139.
[10] K. Vande Casteele, C.F. Van Sumere, J. Chromatogr. 258 (1983) 111–124.
[11] N. Shibuya, Phytochemistry 23 (1984) 2233–2237.
[12] E. Delage, G. Bohuon, A. Baron, J.F. Drilleau, J. Chromatogr. 555 (1991) 125–136.
[13] G.P. Cartoni, F. Coccioli, L. Pontelli, E. Quattrucci, J. Chromatogr. 537 (1991) 93–99.
[14] T.H. Dzido, H.D. Smolarz, J. Chromatogr. A 679 (1994) 59–66.
[15] K. Głowniak, G. Zgórka, M. Kozyra, J. Chromatogr. A 730 (1996) 25–29.
[16] L. Świątek, E. Dombrowicz, Farm. Pol. 40 (1984) 729–732.