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Quality Control of Roots of *Eleutherococcus senticosus* by HPLC

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An HPLC method based on several known methods for the determination of eleutherosides B and E was developed, optimised and validated in terms of linearity, precision (repeatability and intermediate precision on different days and at different concentration levels) and accuracy (recovery). The extraction procedure, the extraction solvent and the extraction yield were evaluated and optimised. A reversed-phase RP-18 column gradient eluted with a two-phase system consisting of phosphoric acid:water (0.5:99.5) and acetonitrile was used to evaluate the samples; detection was at 220 nm. Although eleutherosides B and E are commercially available, they are very costly, and therefore ferulic acid was chosen as external standard. The correction factors for the response of ferulic acid against both eleutherosides were determined and validated. This method, accepted by the European Pharmacopoeia Commission, will be included in the monograph on *Eleutherococcus senticosus* roots to assay the content of eleutherosides B and E. Copyright © 2005 John Wiley & Sons, Ltd.

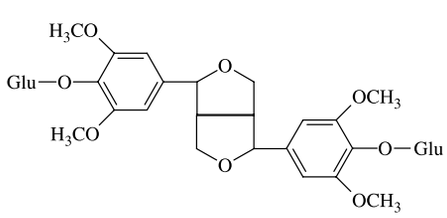
Keywords: HPLC; quality control; method validation; eleutheroside; *Eleutherococcus senticosus* roots.

INTRODUCTION

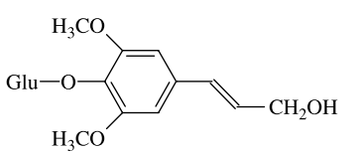
According to the definition in the current edition of the *European Pharmacopoeia*, herbal drugs (Herbal drugs, Plantae medicinales, 01/2002:1433) are mainly whole, fragmented or cut plants, parts of plants, algae, fungi, lichen in an unprocessed state, usually in dried form but sometimes fresh. Common to all of the monographs of the *European Pharmacopoeia*, those on herbal drugs include defined headings, i.e. definition, production, identification, tests, assay and storage. Unless otherwise justified and authorised, herbal drugs must be assayed by an appropriate method. If the components responsible for the activity of the herbal drug are fully or partially known, the assay is based on the determination of these active principles. When this is not the case, markers are used to guarantee constant quality.

The roots and rhizomes of *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim., also known as *Acanthopanax senticosus* and referred to as 'Siberian Ginseng', are used as a tonic and adaptogen. Most of the research concerning the pharmacological effects of *E. senticosus* was initiated in Russia, where the term 'adaptogen' was coined to represent a substance which increases the non-specific resistance of an organism to adverse influences, while generating a normalising action on bodily systems (Farnsworth *et al.*, 1985). The secondary compounds isolated from *Eleutherococcus* include phenylpropanoids (e.g. syringin, caffeic acid, sinapyl alcohol, coniferyl aldehyde), lignans (e.g. sesamin, syringoresinol and its glucoside), saponins (e.g. daucosterol, β-sitosterol, hederasaponin B), coumarins (e.g. isofraxidin and its glucoside), the triterpene betulinic acid and vitamins (e.g. vitamin E) and provitamins (provitamin A, i.e. β-carotene; Davydov and Krikorian, 2000). Characteristic constituents of this plant are the eleutherosides, a group of compounds with widely varied structures (Wagner *et al.*, 1982; Bladt *et al.*, 1990) that are responsible, at least in part, for the adaptogenic activities (Davydov and Krikorian, 2000). Of these eleutherosides, the major compounds eleutheroside E (structure below; syringaresinol di-*O*-β-*D*-glucoside, a lignan) and eleutheroside B (structure below; syringin, a phenylpropane derivative) usually serve as marker compounds in the identification and analysis of *E. senticosus* (Wagner *et al.*, 1982; Bladt *et al.*, 1990; Slacanin *et al.*, 1991; Yat *et al.*, 1998; Kang *et al.*, 2001).

An HPLC method for the determination of eleutherosides B and E has been developed based on known methods (Wagner *et al.*, 1982; Bladt *et al.*, 1990; Slacanin *et al.*, 1991; Yat *et al.*, 1998; Kang *et al.*, 2001) and



Eleutheroside E



Eleutheroside B

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optimised. The extraction procedure, the extraction solvent and the extraction yield were investigated. A reversed-phase HPLC-UV system was used to evaluate the samples. Since pure eleutherosides are expensive and not commonly commercially available, ferulic acid was used as a secondary standard. The method was fully validated according to the ICH guidelines (Text on Validation of Analytical Procedures, 1994; Validation of Analytical Procedures: Methodology, 1996).

EXPERIMENTAL

Solvents, standards and sample

Distilled water was obtained from a Millipore (Brussels, Belgium) water purification system; ethanol (Pro-analysis quality) was purchased from Merck (Darmstadt, Germany) and methanol (HPLC quality) was from Acros Organics (Geel, Belgium). Standards of eleutherosides B (99.47% HPLC purity) and E (98.44% HPLC purity) were purchased from Phytolab (Hamburg, Germany), and ferulic acid (98.95% HPLC purity) was from Acros Organics. A root sample of *Eleutherococcus senticosus* was kindly donated by the World Business Company (Brussels, Belgium).

Equipment

HPLC analyses were carried out using an Agilent (Brussels, Belgium) instrument consisting of a pump model A 1050 and a photodiode array detector (PAD) model A 1040 M HP, equipped with a Gilson 234 automatic injector (Gilson International, Rijswijk, The Netherlands). The second HPLC apparatus used was a Gilson instrument (pump model 322, UV–vis detector model 156) equipped with a Gilson 234 automatic injector. In each case a Merck RP C-18 Lichrospher column (250 × 4.6 mm i.d.; 5 μm) was employed.

Methodology

Test solutions were prepared by adding 30 mL of 50% aqueous ethanol (v/v) to 0.500 g of the powdered drug contained in a round-bottomed flask. After heating under reflux and cooling for 30 min, the extraction mixture was cooled down, filtered through a sintered glass filter and the supernatant liquid collected in a 250 mL round-bottomed flask. This operation was repeated twice on the resulting plant residue and the supernatant liquids were combined and evaporated under reduced pressure to a final volume of ca. 10 mL. The concentrated extract was transferred quantitatively to a 20 mL volumetric flask and made up to 20.0 mL with 50% aqueous ethanol. A reference solution of ferulic acid was prepared by dissolving 10.0 mg of ferulic acid in 50% aqueous methanol and diluting to 20.0 mL with the same solvent; 1.0 mL of this solution was transferred to a 25 mL volumetric flask and diluted to 25.0 mL with 50% aqueous methanol.

The HPLC analyses were performed at ambient temperature: test and reference solutions were filtered through nylon filters (0.45 μm) prior to injection of an appropriate aliquot (20 μL). The mobile phase consisted of phosphoric acid:water (0.5:0.95; solvent A) and acetonitrile (solvent B). The elution programme was: initially 90:10 (A:B) with isocratic elution for 5 min followed by a linear gradient to 80:20 in 22 min, linear gradient to 50:50 in 3 min, isocratic for 5 min, linear gradient to the starting conditions (90:10) in 5 min and isocratic for 5 min (equilibration time). The flow rate was 1.0 mL/min.

Eleutherosides B and E were identified based on their retention times and spectra between 200 and 400 nm; components were quantified at 220 nm. The percentages of eleutherosides were calculated from:

$$\text{Eleutherosides B + E \%} = [(A_B \times C \times 0.73 \times 2)/(A_R \times m)] + [(A_E \times C \times 1.90 \times 2)/(A_R \times m)]$$

where A_B, A_E and A_R are the areas of the peaks associated with eleutheroside B, eleutheroside E and ferulic acid, respectively, C is the concentration of ferulic acid (μg/mL), and m is the mass of the drug (mg).

Validation

The method was validated according to the ICH guidelines on the validation of analytical methods (Text on Validation of Analytical Procedures, 1994; Validation of Analytical Procedures: Methodology, 1996). All results were expressed as percentages, and n represents the number of replicates. For the statistical analysis Excel 2000® (Microsoft) software was used: a 5% level of significance was selected.

Linearity. Reference solutions were prepared at five concentration levels and were injected twice. The concentration levels for eleutherosides B and E and ferulic acid were, respectively, within the range 2.4–38.4, 4.8–38.4 and 2.4–38.8 μg/mL. In order to assess linearity, the least squares line and the correlation coefficient were calculated. The calibration curve obtained was tested on the intercept ($b = 0$) by means of Student's t-tests. To check the goodness of fit of the linear model a lack-of-fit (LOF) test (Miller, 1991) was performed and the residuals were graphically inspected.

Correction factor. Correction factors for eleutherosides B and E against ferulic acid were determined at three concentration levels in triplicate. Solutions containing 20 μg/mL ferulic acid each and about 10, 20 or 40 μg/mL eleutheroside B or E were analysed on two different brands of HPLC equipment. The mean correction factor and the standard deviation were determined.

Precision. The repeatability and the inter-day intermediate precision were determined by analysing six samples (100%) according to the above-described method on three different days. The standard deviation and coefficient of variation were calculated for each day. In order to check whether the results obtained on the different days were significantly different, the results were analysed by means of an ANOVA single factor. Within- and between-days variation coefficients were calculated (Caporal-Gautier *et al.*, 1992). To check the precision of the method over a broad range, six samples weighing half the normally weighed mass (50%) and six samples weighing twice the normally weighed mass (200%) were

analysed according to the method described. The standard deviation and coefficient of variation were calculated for each level. By means of a Cochran's test, the variations at these concentrations were compared with the variation at 100%. In order to check whether the results obtained at the three levels were significantly different, the results were analyzed by means of an ANOVA single factor. Within- and between-level variation coefficients were calculated (Caporal-Gautier *et al.*, 1992).

Accuracy. To half (50%) of the normally weighed mass of root powder (i.e. 250 mg containing 0.233 mg eleutheroside B and 0.243 mg eleutheroside E), an amount equivalent to 35% (ca. 0.170 mg), 70% (ca. 0.340 mg) or 90% (ca. 0.425 mg) of eleutheroside B, or an amount equivalent to 25% (ca. 0.115 mg), 50% (ca. 0.230 mg) or 60% (ca. 0.300 mg) of eleutheroside E was added before the extraction. At each level, samples were prepared in triplicate and each sample was injected twice and analysed according to the method previously described. The mean percentage recovery for both eleutherosides were checked to be equal to 100% by means of Student's *t*-tests.

Specificity. The peaks associated with eleutherosides B and E were identified by retention times and spectra between 200 and 400 nm compared with reference standards.

RESULTS AND DISCUSSION

In order to develop a method suitable for routine quality control of *Eleutherococcus senticosus*, the reference material used as external standard should be readily available at an acceptable price. Since this is not the case for eleutherosides B and E, ferulic acid (4-hydroxy-3-methoxy-cinnamic acid), which is cheap and commercially available, was chosen as the secondary external standard. Ferulic acid could be analysed using the same chromatographic parameters as for the eleutherosides and showed a retention time of about 24 min. The linearity of eleutherosides B and E and ferulic acid was investigated and the results are shown in Table 1. Graphical inspection of the residuals, the LOF test and the correlation coefficients proved the method to be linear for eleutherosides B and E and for ferulic acid in the range tested. The *t*-test on the intercepts revealed that point (0,0) falls within each of the calibration curves.

Correction factors for the differences in response with respect to UV-absorbance at 220 nm of both eleutherosides *vs*. ferulic acid were determined. The mean correction factors (CF = [concentration eleutheroside/area eleutheroside] × [area ferulic acid/concentration ferulic acid] for eleutherosides B and E determined at different concentration levels and on different HPLC equipment, were 0.728 ± 0.027 and 1.897 ± 0.080, respectively.

The HPLC method for the determination of eleutherosides B and E was developed based on known methods (Wagner *et al.*, 1982; Bladt *et al.*, 1990; Slacanin *et al.*, 1991; Yat *et al.*, 1998; Kang *et al.*, 2001) and optimised. The method proposed by Yat *et al.* (1998) using 80% methanol acidified with trifluoroacetic acid to redissolve the residue was validated. According to this method, the root sample contained a mean content of eleutheroside B of 0.0454% (n = 18; six replicates on 3 days), with a between-day coefficient of variance (CV) of 6.20%, and a recovery of 71% (CV = 12%). In the same way, the mean content of eleutheroside E in the sample was 0.0546% (CV = 6.36%) and the recovery was 63% (CV = 7.5%). These validation data indicated that the method was not precise and not accurate for both eleutherosides. The extraction procedure, the extraction solvent and the extraction yield were the investigated further. As shown by Yat *et al.* (1998), the difficulty in this analysis is associated with the redissolution of the eleutherosides after evaporation of the extraction solvent under reduced pressure. The first step in our optimisation of the method was to reduce the volume of the combined extracted fractions to about 10 mL instead of trying to redissolve the totally dry residue with acidified methanol. This change in methodology led to higher yields of eleutherosides B and E, i.e. 0.0579% (n = 3; CV = 8.9%; calculated recovery with respect to the original method = 90.5%) and 0.0650% (n = 3; CV = 11.7%; calculated recovery with respect to the original method = 73.8%), respectively. These results showed, however, that further improvements in the method were necessary.

The next step in the optimisation was the consideration of the composition of the extraction solvent. Different solvent compositions had been reported for the extraction of the eleutherosides: Yat *et al.* (1998) refluxed the ground root powder in 30 mL of 20% aqueous methanol (v/v), whilst Slacanin *et al.* (1991) extracted the plant material twice with 80 mL of 80% aqueous methanol. Based on preliminary work, in-laboratory experience and on the results obtained by Kang *et al.* (2001), the extraction solvent was changed to 50% aqueous ethanol. Furthermore, only one or two extraction cycles (leading to combined extracts) were employed in earlier studies. By analysing separately a third and fourth extraction of the resulting plant residue, it was shown that a third extraction step was necessary in order fully to extract the sample.

An HPLC chromatogram of a root sample of *E. senticosus* obtained using the fully optimised method is depicted in Fig. 1, and the spectra of both eleutherosides

Table 1. Overview of the linearity data of the assay of the eleutherosides B and E and ferulic acid

	Eleutheroside B	Eleutheroside E	Ferulic acid
Correlation coefficient	0.9985	0.9999	0.9999
Slope ± standard error	35519689 ± 613056	15809 ± 93	32506 ± 130
Intercept ± standard error	–31222 ± 14954	937 ± 2192	–3555 ± 1550
Confidence interval (95%)	–64543–2099	–4118–5992	–7129–19
F_{LOF} (F_{crit} = 5.41)	1.7	2.5	1.6
Concentration range (μg/mL)	2.4–38.4	4.2–38.4	2.4–38.8
Number of standards (duplicates)	5	5	5

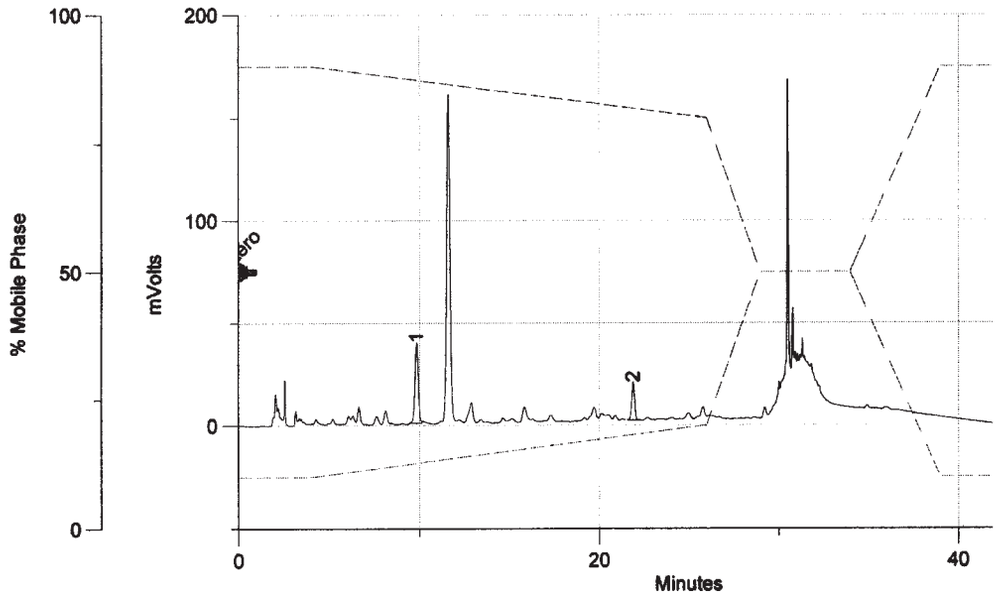

Figure 1. HPLC chromatogram of a root sample of *Eleutherococcus senticosus*. Key to peak identity: **1**, eleutheroside B (retention time 9.86 min); **2**, eleutheroside E (retention time 21.90 min). (For extraction and chromatographic protocols see Experimental section.)

are shown in Fig. 2. Applying the optimised method, the mean contents of eleutherosides B and E were, respectively, 0.0928% and 0.0972% (the concentration of eleutherosides B and E in the sample solutions for injection was about 23 and 24 μg/mL, respectively). In order to investigate the influence of heating the plant material on the stability of the eleutherosides, the analysis was performed by placing the samples, dissolved in 30 mL of 50% aqueous ethanol, on an ultrasonic bath for 1 h instead of refluxing for 30 min. This change in method did not result in higher yields of eleutherosides, on the contrary only about 0.053% (CV = 9.26%) of eleutheroside B and about 0.074% (CV = 5.92%) of eleutheroside E were found. Based on these data, we could conclude that extraction by heating under reflux followed by cooling resulted in the largest amounts of eleutherosides.

The optimised method was fully validated according to ICH guidelines. The validation data (Table 2) show

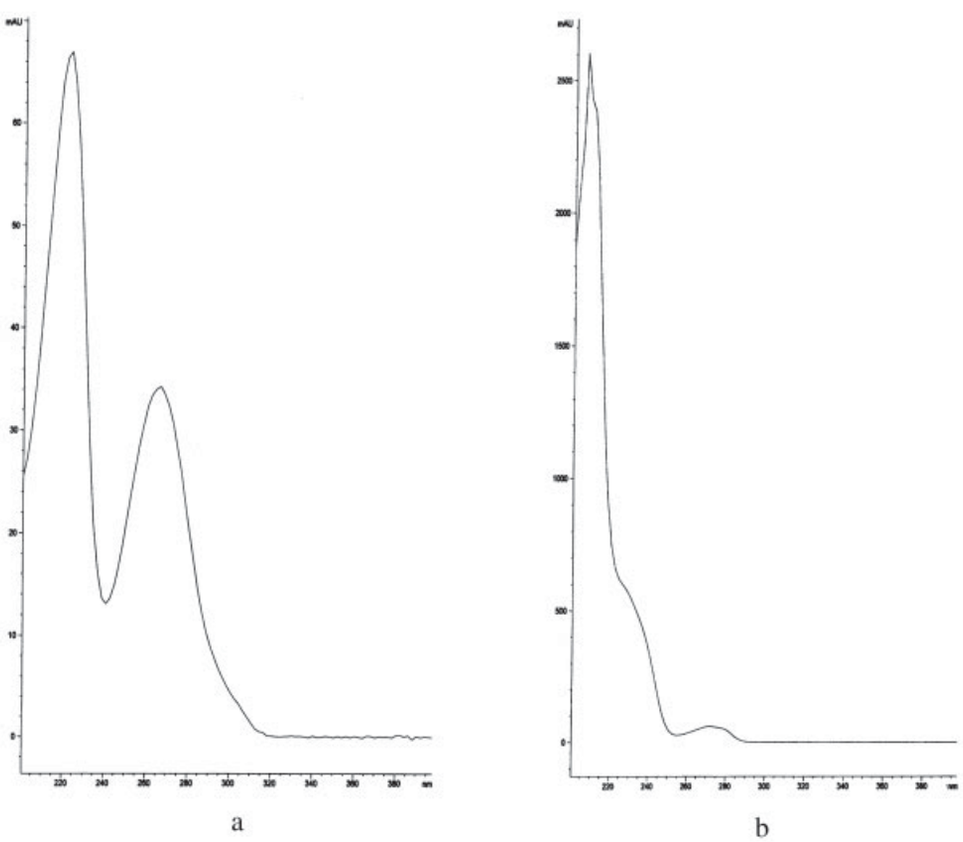

Figure 2. UV spectra (200–400 nm) of (a) eleutheroside B, and (b) eleutheroside E.

Table 2. Validation data for the precision of the final method for the determination of eleutherosides in *Eleutherococcus senticosus* roots

	Eleutheroside B			Eleutheroside E			Total (B + E)		
Precision on different days									
Repeatability									
Number of replicates		6			6			6	
Mean content (%)	0.0927	0.0933	0.0908	0.0939	0.1002	0.0978	0.1866	0.1935	0.1886
RSD% (day 1/day 2/day 3)	3.86	1.97	3.24	1.55	2.42	2.21	2.30	1.98	2.65
Intermediate precision									
Number of days		3			3			3	
Number of replicates		6			6			6	
$RSD\%_{\text{between groups}}$		3.11			3.57			2.56	
F_{calc} (F_{crit} = 3.682)		0.457			13.284			2.348	
Precision on concentration levels									
Repeatability									
Number of replicates		6			6			6	
Mean content (%)(50%/100%/200%)	0.0942	0.0927	0.0925	0.0976	0.1002	0.0977	0.1919	0.1886	0.1901
RSD (%) (50%/100%/200%)	2.52	3.86	1.21	3.01	2.42	1.23	2.70	2.65	0.79
Intermediate precision									
Number of levels		3			3			3	
Number of replicates		6			6			6	
Cochran's test (C_{crit} = 0.707)		0.0648			0.5511			0.4956	
$RSD\%_{\text{between groups}}$		2.76			2.48			2.24	
F_{calc} (F_{crit}= 3.682)		0.756			1.412			0.312	

that the precision of the method was acceptable, i.e. $CV_{\text{between days}}$ of 3.11, 3.57 and 2.56% for eleutherosides B, E and the total, respectively. For eleutheroside B and the total amount of eleutherosides, the ANOVA indicates that, from a statistical point of view, there was no significant difference between the results obtained on three different days. Although the ANOVA was negative for eleutheroside E, the method can be

Table 3. Recovery data for the final method for the determination of eleutherosides in *Eleutherococcus senticosus* roots

	Sample weight (mg)	Determined concentration (%)	Determined concentration (mg)	Added concentration (%)	Added concentration (mg)	Recovery (%)
Eleutheroside B						
1.1	244.9	0.161	0.394	0.069	0.169	98.2
1.2	267.6	0.156	0.417	0.064	0.171	99.4
1.3	258.5	0.161	0.416	0.066	0.171	102.8
2.1	265.4	0.222	0.589	0.128	0.340	100.3
2.2	253.2	0.223	0.565	0.134	0.339	96.5
2.3	259.9	0.224	0.582	0.131	0.340	100.4
3.1	241.9	0.270	0.653	0.176	0.426	100.6
3.2	258.5	0.258	0.667	0.165	0.427	100.3
3.3	256.0	0.247	0.632	0.166	0.425	92.5
Mean						99.0%
Standard deviation						3.0%
RSD (%)						3.0%
$t_{calculated}$						1.02
t_{table}						3.182
Eleutheroside E						
1.1	249.5	0.142	0.354	0.046	0.115	99.2
1.2	246.5	0.147	0.362	0.046	0.113	108.0
1.3	259.9	0.138	0.359	0.044	0.114	94.3
2.1	255.3	0.182	0.465	0.089	0.227	95.7
2.2	245.6	0.189	0.464	0.093	0.228	99.7
2.3	264.2	0.181	0.478	0.086	0.227	97.9
3.1	245.1	0.226	0.554	0.134	0.328	95.9
3.2	259.4	0.211	0.547	0.109	0.283	103.9
3.3	257.1	0.212	0.545	0.110	0.282	103.6
Mean						99.8%
Standard deviation						4.5%
RSD (%)						4.5%
$t_{calculated}$						1.30
t_{table}						3.182

considered precise since the $CV_{between\ days}$ (3.57%) was smaller than 5.69%, the CV calculated by the Horwitz equation (Maas, 1993; Commission of the European Communities, 1998). This $CV_{Horwitz}$ is the maximal variation allowed linked to the concentration of the compound to be determined. Experiments to investigate the precision over a broad range of the method, i.e. between 50% and 200% of the eleutheroside content, were performed and the results are shown in Table 2. Since the Cochran's test was fulfilled for eleutherosides B, E and their total, i.e. $C_{calculated}$ was smaller than $C_{critical}$, the variation of the method can be considered equal for concentration levels within this range. ANOVA analysis of the different concentration levels showed no difference in the results obtained at these levels, which also provides, alongside the recovery test, a good indication of the accuracy of the method. The results of the recovery experiment (Table 3) show that the method was accurate for eleutheroside B (recovery of 99.0%) as well as for eleutheroside E (recovery of 99.8%).

The method was proposed to the European Pharmacopoeia Commission as an assay for root material from *E. senticosus* roots and has been subjected to a collaborative trial and published as part of a monograph proposal (Anonymous, 2002). The final version of the method will be published in the *European Pharmacopoeia* as part of the monograph on *E. senticosus* root for assaying the quality by determining the content on eleutherosides.

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