An HPLC method to determine sennoside A and sennoside B in Sennae fructus and Sennae folium

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ABSTRACT

Introduction. *The current Ph. Eur. monographs for senna pods, senna leaf and senna leaf dry extract standardised describe a photometric assay based on the Bornträger reaction to determine hydroxyanthracene glycosides, calculated as sennoside B. The method is time-consuming, unspecific for sennosides and the precision is not adequate for a modern assay.*

Aim. *The photometric method shall therefore be replaced by a modern HPLC method. About 70 % of the total anthrachinone content in herbal drugs of senna species is due to sennoside A and sennoside B. These substances are therefore suitable for the standardisation of Senna products. The Japanese Pharmacopoeia (JP) already describes an HPLC method to determine sennoside A and sennoside B in the monograph for senna leaf. It uses ion-pair chromatography with tetraheptylammoniumbromide. The procedure described in the monograph has a runtime of 70 min.*

Method. *The adapted and validated method described here uses solid-phase extraction (SPE) which allows a selective sample preparation by using an anion exchange phase. A conventional RP C_{18} column Tosh TSKgel ODS-80TS (4.6 mm x 150 mm), 5 µm, was used as stationary phase and acetonitrile for chromatography R, water R, phosphoric acid R (200:800:1 V/V/V) as mobile phase. The flow rate was 1.2 mL/min, the column temperature 40 °C, the detection wavelength 380 nm, and the injection volume 20 µL. The runtime is 10 min, the chromatogram shows 2 peaks due to sennoside A/B and 2 additional smaller compounds. One of them is rhein-8-O-glucoside.*

Results. *The procedure has been successfully validated according to ICH guidelines. We analysed 6 batches of Senna. The pods (Senna angustifolia) showed a total content of sennoside A and B of 1.74-2.76 % m/m and the content of senna leaves was clearly lower with 1.07-1.19 % m/m, respectively.*

Conclusion. *The suggested method is considered to be suitable to determine sennoside A and sennoside B in senna leaves and senna pods. The consideration is based on the performed validation and on the results for the analysed samples. A short run time and better resolution are clear advantages of the suggested method, compared to other methods.*

KEYWORDS

Sennoside A, sennoside B, senna leaf, senna pods, HPLC, validation.

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1. INTRODUCTION

The European Pharmacopoeia (Ph. Eur.) 8.0 includes monographs for Sennae folium [1], Sennae fructus acutifoliae [2], Sennae fructus angustifoliae [3] and Sennae folii extractum siccum normatum [4]. The respective assay determines the sum of hydroxyanthracene derivatives, calculated as sennoside B. This is a time consuming and unspecific photometric procedure (adapted Bornträger - reaction) and should be replaced by an HPLC method. To this purpose we have adapted an already published rapid and specific HPLC method for the determination of sennoside A and sennoside B by Yamasaki *et al.* [5]. As the method is already very short and simple, there was no optimisation other than adjusting the flow rate from 1 mL/min to 1.2 mL/min. Yamasaki proposed a cold solvent extraction with methanol/aqueous $NaHCO_3$ (2 g/L) 70:30 *V/V* while stirring with a subsequent centrifugation and re-extraction. We found this procedure to be time consuming and replaced it through an ultrasonic extraction with the same extraction solvent. When the drug-solvent ratio was reduced from 20 mg/mL to 2.5 mg/mL, one extraction cycle was found to be equivalent to the method of Yamasaki *et al.*, which employs 2 extraction cycles. A second extraction leads to a 1.5 % increase of the content for sennoside A/B and is therefore not significant [6]. Various samples of senna leaf and senna pods were analysed successfully with this method. Validation was accomplished according to ICH Q2 [7].

2. MATERIALS AND METHODS

2.1. Reagents and samples

99.8 % acetonitrile was from Acros Organics (Nr. AC26827-0025), *sodium hydrogen carbonate R* was from Fluka (No. 88208), the sennoside A (batch No. 2438, article No. 89276, purity p = 96.62 area %, HPLC) and sennoside B (batch No. 7509, article No. 89277, purity p = 98.09 area %, HPLC) mixture were from PhytoLab (Germany). Purified water (0.055 µS/cm) was generated with a Pure Lab Plus UV/UF PL5124 Purification System. The analytical balance model used was a Mettler Toledo XS 204 Delta Range, and the syringe filters, Chromafil PET-45/25, 0.45 µm, were obtained from Macherey Nagel. A Waters Alliance 2695 Separation Module was used as the HPLC apparatus and was equipped with a Waters 2487 DAD detector. A Tosh, TSKgel 80TS, RP C18 (150 mm x 4.6 mm), 5 µm column was used as stationary phase. The vacuum chamber for the SPE was obtained from Macherey Nagel. The SPE cartridges, Oasis MAX® 3cc (60mg), were obtained from Waters. All samples are shown in Table 1. The herbal drugs analysed were delivered by Dixa AG (St. Gallen, Switzerland). All the samples have been analysed by the supplier according to the corresponding Ph. Eur. monographs and were reported to comply with the specifications.

Table 1 – *Senna samples*

Herbal drug	Supplier	Lot No.
Senna leaf Ph. Eur.	Dixa AG	4203602
	Dixa AG	11001809
Senna pods, tinnevelly Ph.Eur.	Dixa AG	4107302
	Dixa AG	3173202
	Dixa AG	3474502
	Dixa AG	2033303

2.2. Liquid chromatography

Test solution. Place 0.125 g of the powdered herbal drug (250) (*2.9.12*) [8] in a 50 mL volumetric flask. Add 45 mL of a mixture of 7 volumes of methanol and 3 volumes of a 0.2 % *m/V* aqueous solution of *sodium hydrogen carbonate R* and extract in an ultrasonic bath for 30 min and fill up to the mark. Filter about 5 mL through a membrane filter (nominal pore size 0.45 µm).

Apply 2.0 mL of the solution to a 3 mL solid phase extraction cartridge (Oasis MAX 3 cc (60 mg) SPE Cartridges from Waters are suitable) previously conditioned with 2 mL of *methanol R* and 2 mL of a 0.2 % aqueous solution of *sodium hydrogen carbonate R*. Wash the cartridge with 2 mL of *water R* and 2 mL of *methanol R* to remove neutral compounds. Wash with 5 mL of a 1 % (*V/V*) *glacial acetic acid R* solution in *methanol R* to remove acidic compounds. Discard the washings. Elute with 2 mL of a mixture of *methanol R*, *water R* and *formic acid R* (70:30:2 *V/V/V*).

Reference solution. Dissolve 2.0 mg of sennoside A and B in 10.0 mL of an aqueous solution of 0.1 % *m/V sodium hydrogen carbonate R*.

Column:

- *size*: *l* = 0.15 m, *Ø* = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (C18 TSKGel, 5 µm, Tosh, Tokyo, Japan is suitable).

Column temperature: 40 °C.

Mobile phase: *acetonitrile R*, *water R*, *phosphoric acid R* (200:800:1 *V/V/V*).

Flow rate: 1.2 mL/min.

Detection: spectrophotometer at 380 nm.

Injection: 20 µL.

Calculation

Calculate the **percentage content of sennoside B** using the following expression:

$$\frac{m_2 \cdot A_1 \cdot 5 \cdot p}{A_2 . m_1}$$

A_1 = area of the peak due to sennoside B obtained with the test solution;

A_2 = area of the peak due to sennoside B obtained with the reference solution;

m_1 = mass of the drug to be examined in the test solution in grams;

m_2 = mass of sennoside B in the reference solution in grams;

p = percentage content of sennoside B.

Calculate the **percentage content of sennoside A** using the following expression:

$$\frac{m_2 \cdot A_1 \cdot 5 \cdot p}{A_2 . m_1}$$

A_1 = area of the peak due to sennoside A obtained with the test solution;

A_2 = area of the peak due to sennoside A obtained with the reference solution;

m_1 = mass of the drug to be examined in the test solution in grams;

m_2 = mass of sennoside A in the reference solution in grams;

p = percentage content of sennoside A.

The method was developed and optimised with the SPE cartridges and the HPLC column mentioned above.

2.3. Validation criteria

2.3.1 Specificity and Selectivity

Specificity for sennoside A and sennoside B shall be demonstrated by calculating the resolution R from a chromatogram obtained with the test solution. The value for R between the respective sennoside and the closest compound has to be 1.5 or greater. The selectivity of the method shall be shown by identification of the peak comparing the respective UV-spectra with the one obtained from the reference solution. Furthermore, the peak purity calculated from the DAD chromatogram has to be 0.995 or greater.

2.3.2 Linearity and range

Linearity and working range shall be demonstrated by 5 point-calibration curves for sennoside A and sennoside B. The coefficients of determination R^2 have to be greater than 0.995. The working range should include ± 50 % of the expected sennoside content in the drug. The expected sennoside content is being estimated by taking into consideration the mean content of all analysed samples.

2.3.3 Accuracy

The accuracy of the method shall be demonstrated by analysing for 6 different samples a mixture containing 1 mL of the test solution and 1 mL of the reference solution. The same test solutions shall be analysed without adding reference solution. The recovery percentage shall be calculated based on these data and shall be in the range from 95 - 105 %.

2.3.4 Repeatability of the method

The repeatability of the whole method shall be demonstrated by preparing and injecting 6 different sample solutions (Lot: 4107302). The RSD (%) of the obtained content must not exceed 5 %.

2.3.5 Intermediate precision

To demonstrate intermediate precision, test solution (Lot: 3173202) shall be injected three times at three different days (0, 1, 9). The RSD of the mean content values for sennoside A and sennoside B must not exceed 2 %.

2.3.6 Robustness

Robustness of the method in case of small changes in various parameters was shown by simulating small modifications. The influence of the variations on sennoside A and B content and retention time was processed and visualised. The method is considered robust with respect to a certain parameter if the deviation of content does not exceed 2 % and the deviation of the retention time does not exceed 30 %. The following modifications were simulated: 18 % acetonitrile in the mobile phase, 22 % acetonitrile in the mobile phase, flow rate 1.1 mL/min, column temperature of 35 °C and 45 °C.

3. RESULTS

3.1. Validation

3.1.1 Specificity

The method described in the current paper meets the defined criterion for specificity both for sennoside A and sennoside B. The resolution R between sennoside A (t_R 7.753 min) and the closest peak (t_R 5.820 min) was 4.1 and therefore greater than 1.5 (see Figure 1). The criterion was also met with respect to sennoside B (t_R 4.307 min) and its closest compound (t_R 5.540 min) because the resolution R was 6.2 and therefore greater than 1.5. The peak purity is 1.000 for sennoside A and 0.9999 for sennoside B.

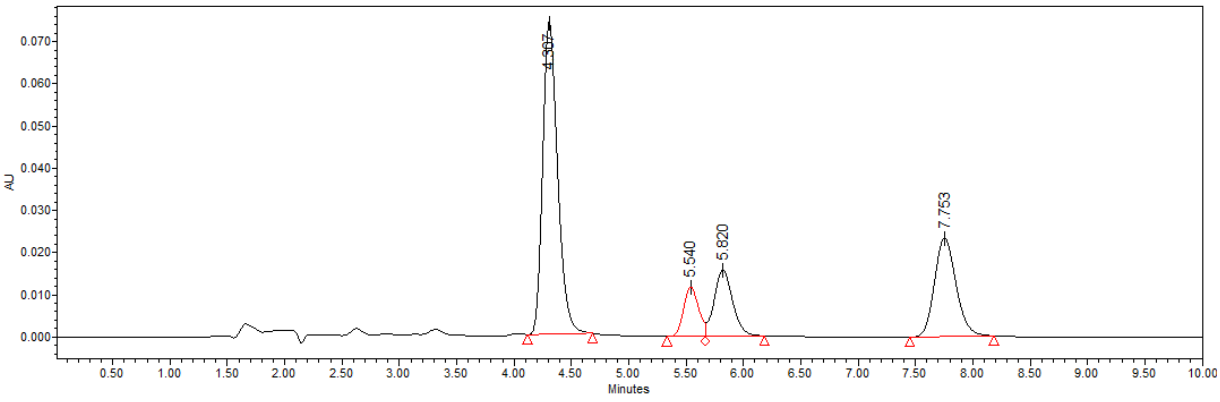
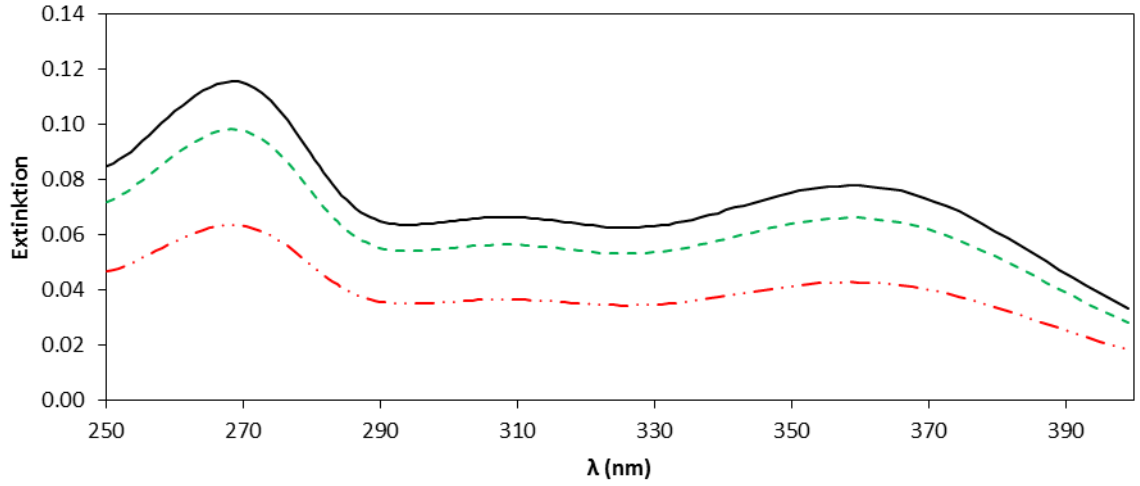


Figure 1 – *Chromatogram obtained by injecting test solution of Sennae fructus*

As shown in Figure 2, the UV spectra for sennoside A and B obtained from the reference solution (black line) are identical to the ones obtained from the test solutions (SA: green, dashed line, SB: red dash-dotted line).



Reference spectrum (black line); spectrum from peak due to SA in test solution (green, dashed line); spectrum from peak due to SB in test solution (red, dash-dotted line).

Figure 2 – *UV spectra from sennoside A and B*

3.1.2 Linearity and range

Linearity was demonstrated across the range defined in Table 2. The calibrated range therefore meets the defined criterion.

Table 2 – *Linear equation, regression coefficient and concentration range for sennoside A and sennoside B*

Analyte	Linear equation	R^2	Concentration range (mg/L)
Sennoside A	y = 11446 x – 5655	0.99997	13.4 - 214
Sennoside B	y = 14452 x – 8725	0.99996	12.8 - 204

3.1.3 Accuracy

Accuracy has been proved by adding 1 mL of the test solution to 1 mL of the reference solution (containing sennoside A and sennoside B). The results are shown in Table 3.

Table 3 – *Recovery of sennoside A and sennoside B, n=6*

Analyte	Range (%)	Recovery (%)	RSD (%)
Sennoside A	-	85.5	4.03
Sennoside B	-	112.8	2.60
Σ sennosides	95 - 105	99.1	3.00

Table 3 shows, that the recovery for sennoside A was rather low (85.5 %) and for sennoside B it is rather high (112.8 %). However, if the sum of both sennosides is taken into consideration, the recovery would be 99.1 % and therefore considered as excellent.

3.1.4 Repeatability of the method

The repeatability of the method was tested and lead to the results presented in Table 4. The RSD was lower than the defined value of 5 % for both sennoside A and sennoside B. Hence, the method is considered repeatable.

Table 4 – *Repeatability for sennoside A and sennoside B, n=6, senna pods, tinnevelly*

Analyte	w mean (% m/m)	VAR	SD (% m/m)	Range SD (%)	RSD (%)	t_R (min)
Sennoside A	0.57	0.0005	0.02	< 5	3.97	8.74
Sennoside B	1.00	0.0014	0.04	< 5	3.78	4.80

3.1.5 Intermediate precision

The defined criterion (RSD t < RSD_{krit}, RSD_{krit} = 2.0 %) for intermediate precision was met, as shown in Table 5.

Table 5 – *Intermediate precision values for sennoside A and sennoside B content*

Analyte	w d_0 (% m/m)	RSD (%)	w d_1 (%)	RSD (%)	w d_9 (%)	RSD (%)	Mean t (%)	RSD t (%)	RSD_{krit} (%)
Sennoside A	1.04	4.49	1.04	2.67	1.07	1.89	1.05	1.35	< 2
Sennoside B	1.75	4.03	1.79	2.13	1.82	0.62	1.79	1.60	< 2

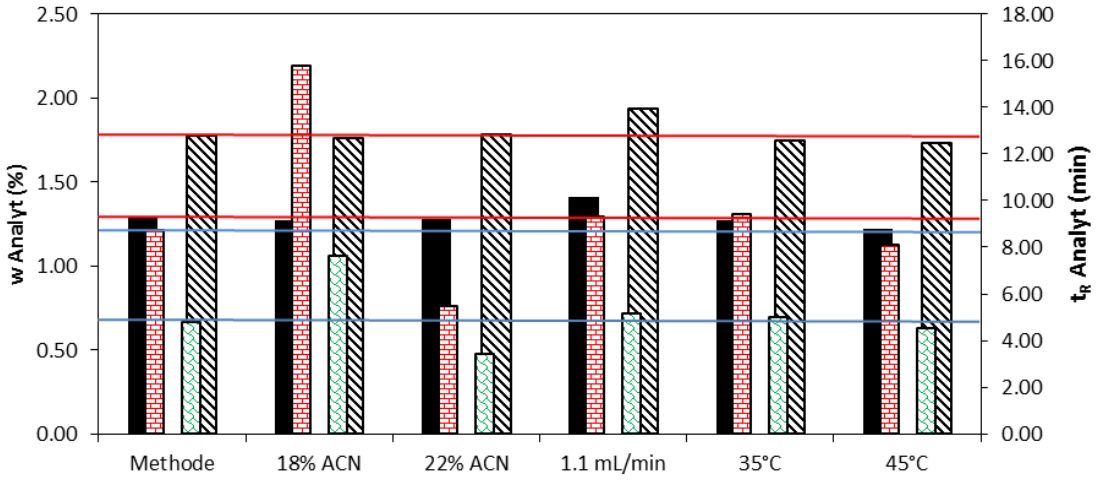
dx = day x; w = weight fraction; mean t= mean for all days.

ICH however suggests to check additional factors such as different analyst, different devices and of course a longer time range [7]. These points could be the goal for further investigations. Based on our experience, there is no evidence that the tests would fail.

3.1.6 Robustness

To prove the robustness of the method both in terms of retention times and content values for sennoside A and sennoside B, 6 separately prepared test solutions from one sample (n = 6) were injected under slightly modified conditions.

Figure 3 shows a comparison of the results obtained under the unmodified conditions with those under modified conditions.



w sennoside A: black bar, w sennoside B: angular filled bar, t_R sennoside A: red dashed bar, t_R sennoside B: angular dashed green bar, w sennosides according to the values obtained from the method: red line, t_R sennosides according to the values obtained from the method: blue line.

Figure 3 – *Robustness of the method with small variations in selected parameters*

The criterion regarding the robustness of a certain parameter with respect to the content of the sennosides (Δ w SA/SB < Δ_{wkrit}) was exceeded when either the flow rate was set to 1.1 mL/min or the column temperature was set to 45 °C.

For the retention times the criterion (Δ t SA/SB < Δ_{tkrit}) was exceeded if the acetonitrile percentage was raised or lowered (see Table 6).

Table 6 - *Relative deviations of sennoside content and retention times of the unmodified method compared with the modified procedures*

Parameter	Δ w SA (%)	Δ w SB (%)	Δ w_{krit} (%)	Δ t SA (%)	Δ t SB (%)	Δ t_{krit} (%)
18 % ACN	1.27	0.54	2.0	81	59	30
22 % ACN	0.74	0.71	2.0	37	29	30
1.1 mL/min	9.62	9.03	2.0	6.4	7.3	30
35 °C	1.32	1.67	2.0	7.7	4.0	30
45 °C	4.89	2.38	2.0	7.1	6.1	30

We used the calibration shown in Table 2 for the runs under modified conditions. This might cause deviations in quantitative analyses.

3.2 Quantitative analysis

The values from the quantitative analysis for sennoside A and sennoside B in the samples are shown in Table 7.

Table 7 – *Results of the quantitative analysis of the samples of Sennae fructus and Sennae folium*

Name	Lot No.	w SA (%)	RSD* (%)	SB (%)	RSD* (%)	∑ HPLC (%)	CoA (%)	W_{min}** JP	W_{min}*** Ph. Eur.
Senna leaf Ph. Eur.	4203602	0.37	2.54	0.70	2.22	1.07	2.80	> 1	> 2.5
	11001809	0.40	2.65	0.79	0.67	1.19	2.90	> 1	> 2.5
Senna pods, tinnevelly Ph. Eur.	4107302	0.59	1.99	1.15	1.17	1.74	2.90	-	> 2.2
	3173202	1.00	3.43	1.74	1.86	2.74	3.60	-	> 2.2
	3474502	0.70	5.32	1.28	3.18	1.98	3.16	-	> 2.2
	2033303	1.00	0.48	1.76	0.55	2.76	4.50	-	> 2.2

* n=3; ** Σ SA and SB, determined by HPLC; *** Hydroxyanthracen glycosides, calculated as sennoside B spectrophotometric method by Ph. Eur.; CoA, spectrophotometric content according to Ph. Eur, from certificate of analysis.

The results show that the sum of the sennoside content obtained with the HPLC method is significantly lower than the values obtained from the photometric procedure according to Ph. Eur. 8.0: for senna pods, tinnevelly (ca. – 40 %) and senna leaf (ca. – 60 %).

According to the values in the certificate of analysis (analysed according to Ph. Eur. by Dixa AG) all samples comply with the specified Ph. Eur. values for the sennoside content. However, the values obtained with the HPLC method comply with the Ph. Eur. specification just for two, (both senna pods) out of six samples.

The JP XVI contains a monograph for Sennae folium [9]. The specification for the minimum content for the sum of sennoside A and sennoside B is ≥ 1 %. Two samples were analysed and were found to comply with this specification.

4. DISCUSSION

The modified method from Yamasaki *et al.* [5,6] is considered to be suitable to determine sennoside A and sennoside B in Sennae folium and Sennae fructus. The consideration is based on the performed validation and on the results for the analysed samples. The short runtime is possible due the preceding solid phase extraction (SPE). The advantage of the SPE is evident when large amounts of samples have to be analysed, e.g. 6 samples can be prepared in 20 min. The chromatogram obtained by this method is simple and easy to process. It shows the respective peaks due to sennoside A (RRT 1.84), sennoside B (RRT 1.00), rhein-8-O-glucoside (RRT 1.28) and one additional up to now unknown compound (RRT 1.38). The resolution R between the sennosides and their closest compounds is greater than 1.5. Due to the strongly increased lipophilic properties of the aglyca rhein and aloeemodin, we suggest to determine them with an alternative method and the corresponding standards [10] (specific method under preparation), if needed at all. The protocol suggested by the JP which uses THpA as ion-pair reagent is characterised by long duration (70 min) [9].

The elaborated method met the pre-defined validation criteria. However, it was found that the method is sensible to relatively small variations in the chromatographic conditions in terms of robustness. This was observed experimentally with other HPLC methods available for sennosides [6]. Based on the accuracy results, it is suspected that isomerisation of sennoside A to sennoside B, would explain the unusual recovery values (sennoside A 85.5 %, sennoside B 112.8 %, average 99.1 %). The isomerisation from sennoside A and G to sennoside B in an aqueous solution of $NaHCO_3$ is reported in the literature, although at 80 °C [11]. The regression equations for sennoside A and sennoside B show similar slopes. We therefore suggest to use a single point calibration with sennoside B and to calculate sennoside A as sennoside B, in

case of implementation of this method into the Ph. Eur. We further want to emphasise that the specified minimum values for the photometric assay according to the Ph. Eur. (2.2 % in senna pods tinnevelly, 2.5 % in senna leaf) are not suitable for the more selective HPLC method. The specifications should be reconsidered and be adapted to the higher selectivity of the method. The JP sets a minimum specification of 1 % sennosides (sum of SA and SB) for senna leaf. Further research and collaborative trials are necessary to define adequate minimum content values for the herbal drugs as well as for the herbal drug preparations (monograph senna leaf dry extract standardised).

The method is also suitable for the analysis of herbal medicinal products as our experience with the fig syrup with senna of the Swiss Pharmacopeia has shown. Validation of the process for the production of herbal medicinal preparations and products of Senna, which are in most cases quite complex, can be accomplished within a reasonable time frame and good repeatability. The mentioned advantages of the method are also important for stability control of herbal drug, herbal drug preparation and herbal drug products of senna.

5. ABBREVIATIONS

t_R	=	retention time
SA	=	sennoside A
SB	=	sennoside B
JP	=	Japanese Pharmacopoeia
Ph. Eur.	=	European Pharmacopoeia
HAG	=	hydroxyanthracene glycosides
w	=	weight fraction in (g/g) or % m/m
SPE	=	solid-phase extraction

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