Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 69 (2006) 418–424
Talanta
www.elsevier.com/locate/talanta

A validation protocol for the HPTLC standardization of herbal products: Application to the determination of acteoside in leaves of *Plantago palmata* Hook. f.s.

G. Biringanine, M.-T. Chiarelli, M. Faes, P. Duez *

Université Libre de Bruxelles (ULB), Institute of Pharmacy, Laboratory of Pharmacognosy, Bromatology and Human Nutrition, CP 205/9, Bd du Triomphe, 1050 Brussels, Belgium

Received 15 July 2005; received in revised form 8 September 2005; accepted 4 October 2005
Available online 11 November 2005

Abstract

Formal validation, that is the study of the analytical performances of a method, is recognized as the best safeguard against the generation and publication of data with low reliability.

Although the topic of HPTLC validations has been largely investigated, there is still a need for a general validation method applicable whenever a blank matrix cannot be reconstituted, notably herbs and their extracts.

This work proposes two validation schemes aiming at generate linearity, accuracy and precision data in a minimal number of HPTLC plates, taking the standardization of *Plantago palmata* as an example with both UV and visible (post-chromatographic derivatization with a sulphuric acid–vanillin reagent) detections. A major problem associated with HPTLC determinations is underlined, namely the low range of linearity which makes spiking studies quite difficult as care must be taken to avoid overloading, whereas keeping the analyte detectable in blank extracts and avoiding spikes too close to endogenous levels. A second problem is the use of general post-chromatographic derivatization reagents that compromise the selectivity of the method by reacting with compounds that may not be resolved from the compound of interest. The use of such reagents is clearly not without danger, especially given the relatively low resolution of planar chromatography.

In conclusion, the retained validation protocol effectively yields the main validation data whereas allowing to pinpoint major analytical drawbacks. It was not possible to simultaneously validate aucubin and acteoside assays as both analytes are present at too different levels/detectabilities.

Keywords: Analytical validation; Aucubin; Acteoside; High performance thin-layer chromatography

1. Introduction

TLC and HPTLC are methods commonly applied for the identification, the assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutriments) [1]. These flexible and cost-effective techniques present the advantage of the simultaneous processing of standards and samples with versatile detection possibilities, including a great variety of post-chromatographic derivatization reagents. The validation of analytical methods is largely recognized as the best safeguard against the generation of unreliable data and is becoming an absolute requirement in many fields. Validation is the process by which it is established, by laboratory studies, that the performance characteristics of an analytical method meet the requirements for the intended applications [2]. Depending on the objective of the analytical procedure, the typical validation characteristics which can be considered through a statistical approach are accuracy, precision, specificity or selectivity, detection limit, quantification limit, linearity and ruggedness [3].

The concept of validation applied to densitometric determinations on high-performance thin-layer chromatography (HPTLC) indeed varies according to the goal of the analysis and the steps required for a formal validation have been thoroughly investigated [1,4–8], notably for purity testing [9], pharmaceutical dosage forms assay [10] and herbals fingerprinting [11]. The

* Corresponding author. Tel.: +32 2 650 5172; fax: +32 2 650 5430.
E-mail address: pduez@ulb.ac.be (P. Duez).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.10.007

assay validations generally rely on the spiking of analytes to a reconstituted blank matrix.

For herbals and their extracts however, blank matrixes can be prepared only for the assay of contaminants (e.g. determination of aflatoxins in wheat [12]) or of components added to the raw extract (e.g. determination of caffeine in some drinks [13]).

For the assay of compounds endogenous to the plant, there is no possibility to reconstitute a blank matrix; some authors have overcome the difficulty by validating methods using standard solutions only [13], an approach yielding however much better results than those which may be forthcoming from a real sample [1]. In fact, such a validation requires spiking of non-blank matrixes, which introduces additional difficulties to the development of the validation scheme; this situation is not unlike those encountered in clinical biology for which more or less standard validation protocols are available [14,15]. However, due to the limited number of spots applicable on a single plate, such protocols cannot be transposed as is to TLC/HPTLC analyses. To our knowledge, no investigation of the steps required for a formal validation has been published and validation schemes tend to vary significantly from author to author [16–18]; performances are difficult to compare, reported data not always allowing to deduce the exact protocol implemented, notably the total number of plates used or of spots applied.

The determination of a phenylethanoid glycoside, acteoside, and of an iridoid, aucubin, in *Plantago palmata* Hook. f. (Plantaginaceae) leaves was selected as a model for developing a validation scheme to measure in only three plates the principal parameters of validation which are linearity, accuracy and precision. Both compounds are present in the plant at different concentration levels, can be extracted with the same solvent and analyzed along the same chromatographic runs. As they can be detected directly by their absorbance in UV, but also in the visible after spraying of a general chromogenic solution, they allow to compare both types of measurements.

Plantago palmata grows in the humid mountain regions of intertropical Africa at 1800–3000 m [19–21] and its leaves have a number of applications in traditional medicine, notably in Burundi, Rwanda and South Kivu (Congo): (i) crushed leaves treat abscesses, wounds, burnings, sting bites; (ii) leaves diluted with water enhance milk secretion and treat woman sterility, abortion menace, eye infections, hemorrhoids, dysentery, gonorrhea, ascaridiasis and hepatitis; (iii) decoctions are a remedy for ascites, hypertension, malaria and stomach ache; (iv) infusions are used for the treatment of pregnancy troubles, colibacillosis and for the improvement of health after disease [21]. The compounds investigated in the present study (Fig. 1) are part of the phenylethanoid glycosides and iridoids of *Plantago*, two groups of metabolites possibly related to their traditional uses and biological activities [18].

2. Material and methods

2.1. Plant

Plantago palmata Hook. f. (Plantaginaceae) seeds were harvested in the Democratic Republic of Congo and grown in a greenhouse (Experimental Garden Jean Massart, Brussels, Belgium); leaves and roots were collected after 3 months of culture, immediately immersed in acetone, dried and powdered. The plant was identified by Professor J. Lejoly in the Laboratory of Systematical Botany and Phytosociology, Free University of Brussels (ULB), Belgium, where a voucher specimen has been deposited. From the ethanol extract of leaves (yield: 8.72% on dried weight), aucubin (0.32%), gardoside (0.005%), 8-epi-loganic acid (0.13%), arborescoside (0.005%) and acteoside (verbascoside) (0.01%) could be isolated in pure form by medium-pressure liquid chromatography, courtesy of Dr. N. Ronsted (Department of Organic Chemistry, The Technical University of Denmark) and identified by NMR spectroscopy. This qualitative composition is similar to previously published data [22].

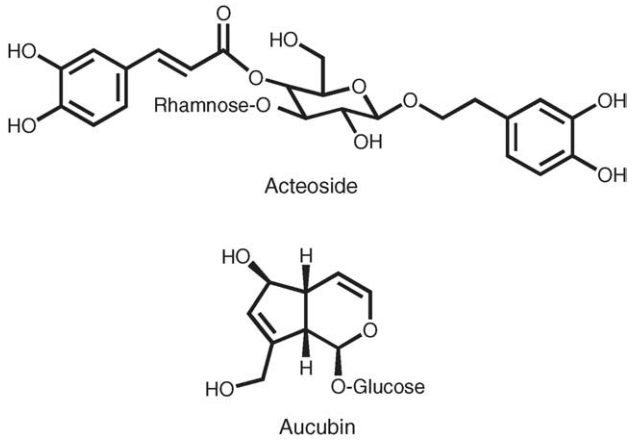

Fig. 1. Structures of acteoside and aucubin.

2.2. Chemicals

Aucubin and acteoside were obtained from Carl Roth, Karlsruhe, Germany. Silicagel 60F_{254} HPTLC plates and solvents were from Merck, Darmstadt, Germany. All other reagents were from Aldrich.

2.3. Extraction and HPTLC conditions

Forty milligram aliquots of leaves powder were spiked as indicated in Table 1 (methanolic solutions), added with methanol up to 10 ml, extracted by agitation (60 min) and centrifuged (2000 × *g*; 15 min); 10 μl of the supernatant were applied on HPTLC plates with a TLC sampler III piloted by the Wincats software 1.3.2 (Camag, Switzerland). Methanolic dilutions of a solution prepared by dissolving 3.75 mg of acteoside and aucubin in 5.0 ml methanol were similarly applied (range 0.3–1.2 and 0.15–1.05 μg/spot, respectively).

After development in ethyl acetate:water:acetic acid:formic acid (67:18:7.5:7.5) [23] and drying at 105 °C for 20 min (Camag TLC Plate Heater III), the plates were measured at 330 nm in reflectance mode with a TLC Scanner III (Wincats 1.3.2, Camag) (Fig. 2); they were then sprayed with 0.5 g vanillin dissolved in methanol:acetic acid:sulphuric acid (85:10:5), heated 20 min at

Table 1
Spike (μg/10 ml) of the samples for the validation study [40.0 mg of *Plantago palmata* powder; estimated endogenous levels, about 1.5% acteoside (600 μg) and 0.25% aucubin (150 μg)]

	Validation scheme 1[a]		Validation scheme 2	
	Acteoside	Aucubin	Acteoside	Aucubin
Sample 1	0	0	0	0
Sample 2	225–400	150	525	150
Sample 3	375–600	300	712.5	300
Sample 4	525–800	450	900	450
Sample 5	675–1000	600	1087.5	600
Sample 6	825–1200	750	–	–

[a] Two experiments were performed with two different spike levels for acteoside (Fig. 3).

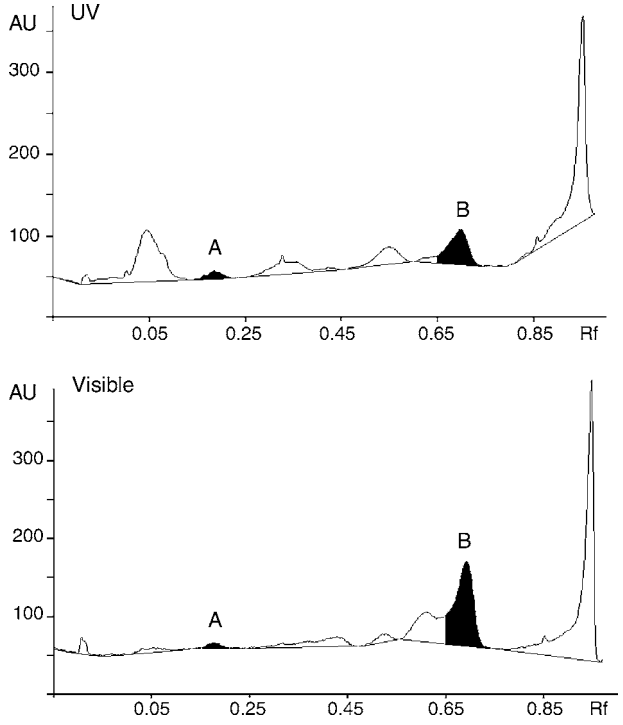

Fig. 2. HPTLC profiles of *Plantago palmata* methanolic extracts (UV: scan at 330 nm; visible: post-chromatographic derivatization with a sulfuric acid vanillin reagent and scan at 520 nm). Peaks: (A) aucubin; (B) acteoside.

105 °C, stabilized 20 min at room temperature in the dark and measured at 520 nm (Fig. 2).

3. Results and discussion

Fig. 2 presents the chromatographic profiles obtained for an unspiked *Plantago palmata* sample, in UV and in visible after spraying of a sulphuric acid–vanillin reagent. UV and visible chromatograms were independently integrated by two different operators to prevent any influence of a known UV chromatographic profile on the integration of the second profile. This avoids preconceived baseline constructions and allows to simulate real conditions in which spraying of a reagent would be the only way to detect compounds.

3.1. Validation scheme

Computing and comparing standard curves and spiked samples curves is an elegant and efficient way to derive linearity, accuracy and precision data from a minimal number of experiments; for HPLC, this method has been showed to allow compliance with ICH validation requirements [24].

The limited number of spots applicable on TLC prompted us to investigate two possible validation schemes (Table 2) to yield all this information while using a minimal number of plates.

We propose a first scheme using two different TLC plates for each experiment, one dedicated to standard solutions (triplicate six-levels calibration curves) and the other to spiked samples (triplicate six-levels of spike linearity curves). An additional standard point is introduced in triplicate on each plate as internal standardization to correct for eventual between-plates variations.

A second scheme (Table 2) proposes to use only one TLC plate for both standard solutions (duplicate five-levels calibration curve) and spiked samples (duplicate five-levels spike linearity curve). As previously, an additional standard point is introduced in triplicate on the plate as internal standardization to correct for between-plates variations. The complete experiment was repeated three times.

Trials with validation scheme 1 proved unsuccessful; although calibration and spiked samples response curves are essentially parallel (Fig. 3), they were difficult to reproduce from experiment to experiment for both analytes and measurement types (UV or visible). The correction of measurements by an internal standardization, as devised in Table 2, was not sufficient to remove these quite considerable differences; these may be due to quenching from residual solvents (UV measurements) or to differences in the development of the chromogenic reaction (visible measurements). As validation scheme 1 in fact investigates spiked samples and standard curves on different plates, this approach appears too prone to variations in the experimen-

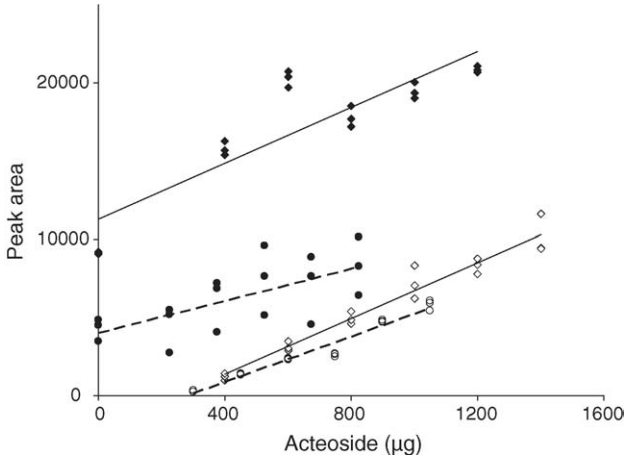

Fig. 3. Example of linearities measured using validation scheme 1 (data from two experiments; spots application according to Table 2) (acteoside; UV: scan at 330 nm): (♦) spiked *Plantago* samples (experiment 1), (◊) standard solutions (experiment 1), (●) spiked *Plantago* samples (experiment 2), (○) standard solutions (experiment 2).

Table 2
Proposed schemes of spots application for the study of linearity, accuracy and precision

Validation scheme	Total number of spots	Number of points for the standard curve	Number of points for the spiked samples curve	Internal standardization[a]
A (two plates[b])	21	Six concentrations, each repeated three times	0	Median standard, applied in triplicate
	21	0	Six concentrations, each repeated three times	Median standard, applied in triplicate
B (one plate[b])	23	Five concentrations, each repeated two times	Five concentrations, each repeated two times	Median standard, applied in triplicate

[a] Used for between-plates standardization (e.g. for a given plate, divide all measured areas by the mean area of the corrector's three spots).
[b] Number of HPTLC plates required for each experiment (the complete validation requires a minimum of three experiments).

tal conditions and was then discarded in favor of scheme 2. Even more thorough standardization of chromatographic conditions (drying, spraying and heating) was also applied for the subsequent experiments.

3.2. Linearity of the HPTLC densitometric method

Fig. 4 and Table 3 present the concentration versus detector response curve for both calibrators and spiked plant extracts according to validation scheme 2 for acteoside and aucubin, with detection in UV and visible (post-chromatographic derivatization).

A relationship "detector response" versus "concentration" could be demonstrated in the investigated ranges (Table 3, slope significance tests) for both analytes in UV and visible detection. The lack of fit test is not conclusive; however this test is notoriously unreliable and a visual assessment of linearity is a preferred technique [15]; in the present case, a linear relationship is acceptable. The slopes are parallel, justifying the use of pure standards for daily work.

The regression lines of standards do not pass through the origin (p of the t-test "intercept $\neq 0$" < 0.05); this is generally the case in TLC reflectance measurements and will lead to systematic errors with increasing difference between the signal of the analysis and that of the standard [25]. A proposed solution consists in using multiple level calibration each time the procedure is carried out [10].

For this study, spike was at the earliest possible analytical step; given the high cost of standards, the only possibility to enrich samples in suitable concentrations was to reduce

Table 3
Linearity studied using validation scheme 2 (regression on data from the three experiments; spots application according to Table 2; measurements corrected by internal standardization; UV: scan at 330 nm; visible: post-chromatographic derivatization with a sulphuric acid–vanillin reagent and scan at 520 nm)

Compound	Acteoside (UV)	Acteoside (visible)	Aucubin (UV)	Aucubin (visible)
Standard solutions				
Determination coefficient r^2	0.8608	0.8750	0.9202	0.7861
Slope ± standard deviation (RSD%)	0.00156 ± 0.00013 (8.3%)	0.00157 ± 0.00012 (7.6%)	0.00165 ± 0.0001 (6.1%)	0.00123 ± 0.0001 (8.1%)
Intercept ± standard deviation (RSD%)	−0.234 ± 0.11 (47%)	−0.301 ± 0.10 (33%)	0.040 ± 0.066 (165%)	0.222 ± 0.088 (40%)
p for the slope significance F-test	$3.34 \times 10^{-12***}$	$8.63 \times 10^{-13***}$	$3.10 \times 10^{-15***}$	$7.50 \times 10^{-10***}$
p for the lack of fit F-test	0.001^{**}	0.008^{**}	0.111^{NS}	0.069^{NS}
Spiked *Plantago palmata*				
Determination coefficient r^2	0.8485	0.7214	0.4076	0.5241
Slope ± standard deviation (RSD%)	0.00133 ± 0.00012 (9%)	0.00155 ± 0.0002 (13%)	0.00128 ± 0.0003 (23%)	0.00140 ± 0.0003 (21%)
Intercept ± standard deviation (RSD%)	0.707 ± 0.08 (11%)	1.336 ± 0.14 (11%)	0.313 ± 0.10 (32%)	0.229 ± 0.086 (38%)
p for the slope significance F-test	$1.75 \times 10^{-10***}$	$1.53 \times 10^{-7***}$	$7.88 \times 10^{-4***}$	$6.29 \times 10^{-5***}$
p for the lack of fit F-test	$8.06 \times 10^{-6***}$	0.136^{NS}	$2.05 \times 10^{-9***}$	$2.94 \times 10^{-10***}$
p for the parallel slopes t-test (standard solutions vs. spiked *Plantago palmata*)	0.206^{NS}	0.923^{NS}	0.287^{NS}	0.58^{NS}
p for the difference in intercept t-test (standard solutions vs. spiked *Plantago palmata*)	$6.72 \times 10^{-9***}$	9.52×10^{-13}	0.028^{**}	0.951^{NS}
Level in *Plantago palmata* (determined as the y-intercept of the spiked samples regression line)	1.32%	2.16%	0.61%	0.41%
Level in *Plantago palmata* (determined by computation vs. the standard regression line)	1.44 ± 0.09%	2.66 ± 0.24%	0.16 ± 0.13%	n.a.[a]

NS: non-significant at $p > 0.05$.
[a] No difference in intercept between the regression lines standard solutions and spiked *Plantago palmata* ($p = 0.951$).
** Highly significant at $p < 0.01$.
*** Very highly significant at $p < 0.001$.

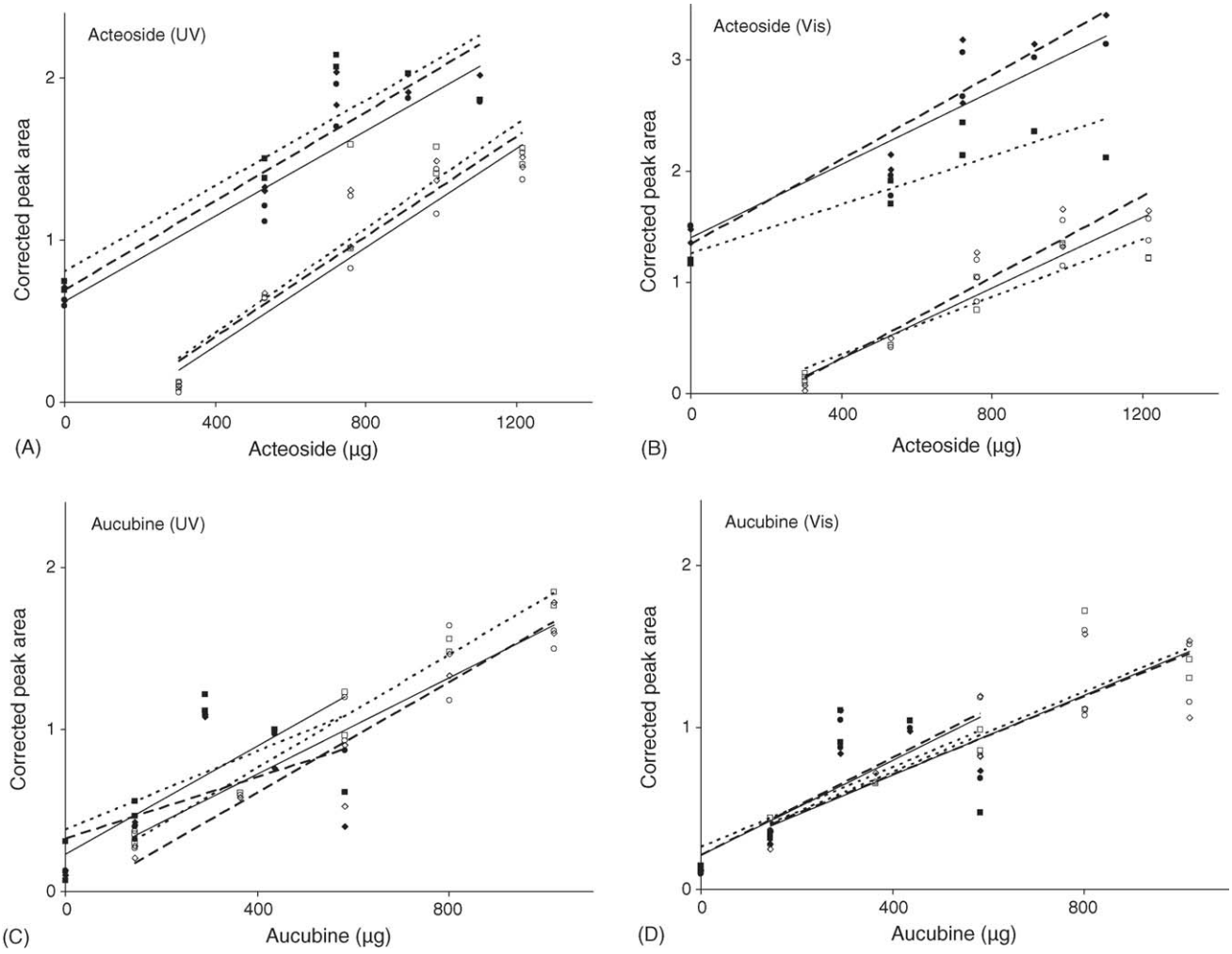

Fig. 4. Linearities measured using validation scheme 2 (data from three experiments; spots application according to Table 2; measurements corrected by internal standardization; UV: scan at 330 nm; visible: post-chromatographic derivatization with a sulfuric acid vanillin reagent and scan at 520 nm; three obvious outliers have been discarded): (⧫, ●, ■) spiked *Plantago palmata* samples (days 1–3), (◊, ○, □) standard solutions (days 1–3), (—, - - -, · · ·) regression lines for spiked *Plantago palmata* samples and standard solutions (days 1–3).

the sample size and solvent volumes; unavoidable small variations in aliquoted *Plantago palmata* powder and in sample homogeneity are reflected in spiked calibration curves and determination coefficients.

For acteoside, there is a significant difference in found levels according to the detection method. In fact, the use of a general chromogenic reagent (sulphuric acid–vanillin reagent) reveals a strong underlying contaminant that was barely detectable in UV. This general problem of wide-ranging spray reagents is most likely overlooked when there is no other detection possibility. In this case, and based on the UV profile, a skimmed integration appears suited to overlook the problem; but without an a priori knowledge of the profile, this rare integration method would most probably be not chosen.

Aucubin presents the double problem of a low endogenous level and a low detectability in UV and in visible. Whereas the peak is clearly over the detection limit, it was not possible to validate the accuracy and precision of the assay as intercepts of both regression lines are very near (UV) or statistically identical (visible).

3.3. Accuracy of the HPTLC densitometric method for the determination of acteoside

For accuracy evaluation (Table 4), the blank levels were estimated from the spiked *Plantago* regression curves as the x intercept and subtracted from found levels [26]. The probability for the F-ratio "variance between concentration levels" to "variance within concentration levels" was then computed to ascertain whether the variations of observations between the concentration levels are due to experimental errors. The mean recovery was then computed along with its confidence interval for a probability level of 0.05. For acteoside UV measurements, the mean relative recovery is around 100%. A systematically lower recovery at the higher spike level can be explained by a probable saturation of absorbance readings; this also explains the significance of the F-ratio ($p < 0.001$). For acteoside visible measurements, a bias statistically significant at the level 0.05 (2.0–39.8%) is observed; this probably arises from the underlying contaminant revealed by the spray reagent. The relatively high standard deviations observed for accuracies come from the

Table 4
Accuracy study for acteoside (parameters as in Table 3)

	Spike level (μg)	Relative recovery (%) (mean ± S.D.)	
		UV	Visible
	532	86.2 ± 16.1	103.6 ± 19.2
	722	121.1 ± 14.5	143.8 ± 34.3
	912	94.5 ± 5.6	124.8 ± 29.5
	1102	76.8 ± 5.4[a]	105.9 ± 39.2
p[a] for the Bartlett χ^2-test (homoscedasticity)		0.293^{NS}	0.617^{NS}
p[a] for the F-test ratio "variance between-levels" to "variance within-levels"		$7.89 \times 10^{-4***}$	0.144^{NS}
Mean relative recovery (%) ± S.D.		97.7 ± 21.6	120.9 ± 32.7
Mean recovery confidence interval (%)		85.2–110.1	102.0–139.8

NS: non-significant at $p > 0.05$.
[a] This systematically lower recovery can be explained by a probable saturation of absorbance readings.
*** Very highly significant at $p < 0.001$.

Table 5
Precision study for acteoside: two-way ANOVA with repetition; two random factors ("*level*" and "*plate*") (three different concentration levels have been analyzed in duplicate on three different plates)

	Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (F)	p	RSD% (within-plate)	RSD% (total)
Acteoside UV	Plate	2	95.39	47.69	4.21	0.104^{NS}	5.99	6.49
	Sample	2	20423.70	10211.85	900.59	$4.91 \times 10^{-6***}$		
	Plate × sample (interaction)	4	45.36	11.34	0.32	0.855^{NS}		
	Residual (error)	9	315.16	35.02				
	Total	17	20879.61					
Acteoside visible	Plate	2	453.81	226.91	1.97	0.253^{NS}	8.23	8.74
	Sample	2	21260.5	10630.32	92.44	$4.49 \times 10^{-4***}$		
	Plate × sample (interaction)	4	459.99	115.00	0.79	0.559^{NS}		
	Residual (error)	9	1306.12	145.12				
	Total	17	23480.56					

NS: non-significant at $p > 0.05$.
*** Very highly significant at $p < 0.001$.

method precision but mainly from the difficulty inherent to sample aliquoting discussed here above.

3.4. Precision of the whole analytical procedure for the determination of acteoside

The data from spiked samples were analyzed for acteoside by a two-way ANOVA with repetition, considering two random effects, "*sample* (*concentration*)" and "*plate*"; the within-plate and total ("between-plates") variations for the whole analytical procedure were computed.

Table 5 details the components of the variance for the precision data. The acteoside precision was not influenced by the factor "*chromatographic plate*" and there was no interaction "*plate*" × "*spike level*". The fair precision allowed to differentiate the levels of the different spiked samples without influence of the factor "*chromatographic plate*". The total precision (RSD%) of the determination was 6.5% and 8.7% for UV and visible measurements respectively. These can be compared with a very recent work [17] on the HPTLC determination of acteoside in *Plantago lanceolata* (UV detection at 334 nm; mobile phase, ethyle acetate:water:formic acid 90:5:5). These authors measured the RSD at one level of concentration, from six spots of a standard solution repeated on three plates (one plate per day) (RSD% of the daily averages: 2%). This value is probably more "presentable" than the RSD% measured in the present work, but it clearly does not represent the variability that can be expected by applying the analytical method to real samples.

4. Conclusion

Although the topic of HPTLC validations has been largely investigated for purity testing, pharmaceutical dosage forms assay and herbals fingerprinting, there is still a need for a general validation method for those cases in which a blank matrix cannot be reconstituted, notably herbs and their extracts. Two of the problems associated with HPTLC determinations have been underlined in the present study, namely the low range of linearity (generally 1–2 log only) and the use of general chromogenic reagents. The low range of linearity makes spiking

studies quite difficult as much care must be taken to avoid overloading, whereas keeping the analyte detectable in blank extracts and avoiding spikes too close to endogenous levels. The study demonstrates that it is not possible to simultaneously validate aucubin and acteoside assays as both analytes are present at too different levels/detectabilities. The validation of aucubin determination remains feasible, but would require higher amounts of the *Plantago* sample (e.g. 100–150 mg) that would give so high a signal for acteoside that further spike of the latter analyte would not be possible.

Specificity ("*peak purity*") is always difficult to assess in any chromatographic system. In the visible light determination of acteoside, the use of a sulphuric acid–vanillin reagent reveals an additional compound present in the peak; detection of the interference and further improvement of the chromatographic separation is possible as this underlying compound is partly resolved. But this may not always be the case and the use of such wide-ranging post-chromatographic derivatization reagents is obviously not without danger, especially given the relatively low resolution of planar chromatography.

In the case of *Plantago*, the proposed chromatographic conditions should clearly be further optimized; the present system could however be accepted but to the condition to measure acteoside in UV only.

The validation protocol (scheme 2) proposed here is quite versatile and gives much information in a very limited number of experiments; its application however requires a preliminary test to assess both detectability and spiking range.

Acknowledgements

We are grateful to Dr. A. Kumps for helpful statistical discussions and M.O. Vaillant for maintaining good conditions of work in the laboratory.

References

[1] G. Szepesi, J. Planar Chromatogr.-Mod. TLC 6 (1993) 187.
[2] USP XXV, Validation of Compendial Methods ⟨1225⟩, The Pharmacopoeia of the United States of America, vol. XXV, United States Pharmacopoeial Convention Inc., Rockville, MD, 2002, pp. 2256–2259.
[3] Commission of the European Communities – Directorate General Industry, CPMP Working Party on Quality of Medicinal Products, III/5626/93-EN, 1993.
[4] B. Renger, J. AOAC Int. 81 (1998) 333.
[5] K. Ferenczi-Fodor, Z. Vegh, A. Nagy-Turak, B. Renger, M. Zeller, J. AOAC Int. 84 (2001) 1265.
[6] K. Ferenczi-Fodor, Z. Vegh, Z. Pap-Sziklay, J. Planar Chromatogr.-Mod. TLC 6 (1993) 198.
[7] G. Szepesi, J. Planar Chromatogr.-Mod. TLC 6 (1993) 259.
[8] M. Prosek, A. Golc-Wondra, I. Vovk, J. Chromatogr. Sci. 40 (2002) 598.
[9] K. Ferenczi-Fodor, A. Nagy-Turak, Z. Vegh, J. Planar Chromatogr.-Mod. TLC 8 (1995) 349.
[10] S.W. Sun, H. Fabre, J. Liquid Chromatogr. 17 (1994) 433.
[11] K. Koll, E. Reich, A. Blatter, M. Veit, J. AOAC Int. 86 (2003) 909.
[12] E. Papp, A. Farkas, K.H. Otta, E. Mincsovics, J. Planar Chromatogr.-Mod. TLC 13 (2000) 328.
[13] I. Vovk, A. Golc-Wondra, M. Prosek, J. Planar Chromatogr.-Mod. TLC 10 (1997) 416.
[14] E.K. Schultz, Selection and interpretation of laboratory procedures, in: C.A. Burtis, R.A. Edward (Eds.), Tietz Textbook of Clinical Chemistry, 3rd ed., W.B. Saunders Co., Philadelphia, 1999.
[15] NCCLS, NCCLS Evaluation Protocols SC1-B, National Committee for Clinical Laboratory Standards, Villanova, PA, 1992.
[16] R.J. Vanhaelen-Fastre, M.L. Faes, M.H. Vanhaelen, J. Chromatogr. A 868 (2000) 269.
[17] A. Umek, A. Rupert, A. Mlinaric, J. Kac, J. Planar Chromatogr.-Mod. TLC 18 (2005) 147.
[18] R. Slaveska-raicki, V. Rafajlovska, V. Rizova, I. Spirevska, J. Planar Chromatogr.-Mod. TLC 16 (2003) 396.
[19] G. Troupin, Flore du Rwanda, Spermatophytes, Agence de Coopération Culturelle et Technique, Paris, 1985, p. 506.
[20] S. Lisowski, Malaisse, J.J. Symoens, Flore d'Afrique Centrale, Zaire-Rwanda-Burundi, Spermatophytes, Plantaginaceae, Jardin Botanique National de Belgique, Université Nationale du Zaire, Lubumbashi, Bruxelles, 1972, pp. 1–5.
[21] G. Defour, Eléments d'identification de 400 plantes médicinales et vétérinaires du Bushi, 1e partie, Bandari, Bukavu-Zaire, 1995, pp. 10–11.
[22] N. Ronsted, H. Franzyk, P. Molgaard, J.W. Jaroszewki, S.R. Jensen, Plant Syst. Evol. 242 (2003) 63.
[23] Council of Europe, *Plantaginis lanceolatae* folium, European Pharmacopoeia, 5 ed., Council of Europe, Strasbourg, 2005.
[24] J. Caporal-Gautier, J.M. Nivet, P. Algranti, M. Guilloteau, M. Histe, M. Lallier, J.J. N'Guyen-Huu, R. Russotto, S.T.P. Pharma Prat. 2 (1992) 205.
[25] S. Ebel, Quantitation in TLC, in: F. Geiss (Ed.), Fundamentals of Thin-layer Chromatography, Dr. Alfred Hüthig Verlag, Heidelberg, 1987, pp. 420–436.
[26] P. Duez, M. Helson, T.I. Somé, J. Dubois, M. Hanocq, Free Radic. Res. 33 (2000) 243.