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Validation, transfer and measurement uncertainty estimation of an HPLC–UV method for the quantification of artemisinin in hydro alcoholic extracts of *Artemisia annua* L.

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ABSTRACT

Malaria is the world's most important parasitic infection with 500 millions cases annually and almost 2 millions death per year. This disease is more present in Sub-Saharan Africa where 90% of the infections are found. Artemisinin and its semi synthetic derivatives (artemether, artesunate) have actually the most powerful activity on malaria, even in its complicated forms and resistance cases.

Various methods have been proposed for detection and quantification of artemisinin in *Artemisia annua L.* by HPLC–UV, but the plant extracts used for this quantification were extracts obtained with organic solvents (toluene, petroleum ether, hexane). To be able to use crude *A. annua* extracts prepared at low cost to formulate antipaludic drugs, we chose the use of a mixture of water and ethanol as solvent of extraction, but no adequate analytical method for this kind of extracts is published.

The main objectives of this work were first to develop an analytical method for artemisinin quantification in hydro alcoholic extracts of *A. annua.* Second, this method had to be thoroughly validated by the research and development laboratory and, third, the transfer of this method to the routine laboratory had to be demonstrated. The final aim was to compare the estimation of measurement uncertainty obtained during the method validation with validation standards to measurement uncertainty estimates obtained during the method transfer study with real samples.

The method was validated following the accuracy profile methodology and was found to be accurate in the concentration range of 10.0–54.0 μg/ml with CV < 8%. Limit of detection and of quantification were 2.73 and 10.0 μg/ml, respectively. The method was then successfully transferred to a laboratory in Benin by showing that the quality of the results that it will generate during routine application of the method is sufficient. Finally, the measurement uncertainty of the method was estimated from the validation experiments as well as from the transfer study with authentic unspiked samples of *A. annua.* The comparison of these measurement uncertainty estimations showed that they were coherent. It confirmed thus that the estimation of measurement uncertainty from validation experiments predicts well the measurement uncertainty of real routine samples. This analytical method was thus shown to be convenient for routine analysis of hydro alcoholic extracts of *A. annua* in Benin.

1. Introduction

Malaria is the world's most important parasitic infection with 500 millions cases annually and almost 2 millions death per year. This disease is mainly present in Sub-Saharan Africa where 90% of the infections are found [1].

Despite tremendous efforts for the control of malaria, the global morbidity and mortality has not changed over the last 50 years. In fact, different drugs are used for the treatment of malaria, like chloroquine, pyrimethamine and mefloquine but resistance was found for these products.

Artemisia annua L. (annual wormwood) was used in traditional Chinese medicine for the treatment of febrile diseases and malaria for many centuries. The active compound, artemisinin, was isolated by Chinese researchers in the early 1970s [2]. In the

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last two decades, artemisinin and its semi synthetic derivatives (artemether, artesunate) have been established as safe and effective antimalarials [3]. Indeed, these molecules showed so far the most powerful activity on malaria, even in its complicated forms and resistance cases.

Artemisinin is a sesquiterpene lactone with a peroxide bridge. Various methods were proposed in the past for its detection and quantification such as thin layer chromatography (TLC) [4,5], high-performance liquid chromatography with UV detection (HPLC–UV) [6], HPLC with electrochemical detection (HPLC–ECD) [7,8], HPLC with evaporative light scattering detection (HPLC–ELSD) [9,10], gas chromatography with mass spectrometric detection (GC–MS) [11], GC with flame ionization detection (GC–FID) [12], and enzyme-linked immunosorbant assay (ELISA) [13]. Mass spectrometry has high sensitivity compared to other detectors and the benefit of molecule identity confirmation through its major ions, but requires great investment and expertise. Detection of artemisinin by UV is the most affordable especially in poor countries like Benin, but artemisinin must be derivatized due to the lack of chromophores. The monographs of World Health Organization (WHO) and International Pharmacopeia [14,15] describe an HPLC method without derivatization using UV detection at 214 nm and dihydroartemisinin as an internal standard. This HPLC–UV analysis method is often reported to be used not only for pure artemisinin, but also for extracts, where its validity has not been proven; indeed we can have problems of interferences because extracts contain a lot of substances. Many authors discarded the WHO HPLC method on the basis of very low UV absorbance of artemisinin [16–18].

The method developed by Zhao and Zeng [6] for the quantification of artemisinin in *A. annua* by HPLC–UV after derivatization has been modified through the years by various authors [17,19–22] as summarized in Table 1. But the plant extracts used for these quantifications were obtained with organic solvents such as toluene, n-hexane, chloroform or petroleum ether which is the most currently used [18].

Aiming at using crude extracts of *A. annua* prepared at lower cost to formulate antipaludic drugs, the use of a mixture of water and ethanol as extraction solvent was selected. The available methods, including the one of Zhao and Zeng [6], failed to quantify artemisinin in this hydro alcoholic extracts. In fact, problems of overpressure on HPLC column with the method of Zhao and Zeng were observed due to the presence of hydrophilic substances in greater amounts than the extracts obtained with organic solvents. Furthermore, the absence of interferences due to these compounds had to be verified. The interest of using hydro-alcoholic extracts is to propose a low cost alternative to artemisinin based treatments or a standardized alternative to treatments with *A. annua* teas for local populations. In fact, the preparation of teas has been shown to lead to very low and variable artemisinin contents ([18,11]) and is difficult to standardize. Compared to organic solvents (toluene, n-hexane, chloroform or petroleum ether) used to extract artemisinin, the use of hydro-alcoholic mixtures have the advantage of being much cheaper, accessible and not toxic.

Among all the methods previously proposed to quantify artemisinin, HPLC–UV is the cheaper and the most accessible one after TLC method in poor countries like Benin. The latter has been applied to quantify artemisinin in our extracts, but the results were not reproducible (data not shown). This lack of precision was also observed by Marchese et al. (2001) [19] who concluded that TLC over-estimated the contents of artemisinin after comparison of the TLC results to those obtained by HPLC on the same samples.

Thus, the objectives of this work are first to develop an improved analytical method based on the method of Zhao and Zeng for artemisinin quantification in hydro alcoholic extracts of *A. annua* as none of the previously developed ones fulfilled this aim adequately. Second, in order to ensure that reliable results will be obtained by this method, it has to be fully validated. To do so the accuracy profile approach was implemented [23,24]. A third objective was to demonstrate the transferability of this method from a Belgian laboratory to a laboratory in Benin where routine analyses are planned to be performed. The method transfer is assessed using the methodology of Dewé et al. [25] with samples coming from the extracts of the same plant material. Finally, to allow adequate interpretation of the analytical results obtained with the developed method, measurement uncertainty was estimated from the validation experiments. This estimation was, for the first time, further compared to the measurement uncertainties obtained from the method transfer study to evaluate whether a validation study using spiked validation standards could adequately estimate measurement uncertainty of real samples of extracts of *A. annua*.

2. Materials and methods

2.1. Materials: plants, chemicals and reagents

The seedlings *of A. annua* used in this study were provided by the Laboratory of Applied Ecology (LEA) of the Agronomic Faculty of Sciences of Abomey Calavi University in Benin. In fact, this Asian plant had been acclimatized and set in culture in Benin. The seeds used were obtained from Anamed (Winnenden, Germany).

Standard artemisinin (98%) was purchased from Sigma–Aldrich (Darmstadt, Germany). Ethanol (Ph Eur 96%) was acquired at VWR (Fontenay-sous-bois, France). Methanol was HPLC-Grade and was purchased, with KOH from VWR. All the other reagents (monopotassic and dipotassic phosphate and phosphoric acid 98%) were of analytical grade and acquired at Merck (Darmstadt, Germany) and Alfa Aesar (Karlsruhe, Germany), respectively.

2.2. Methods

2.2.1. Treatment and analysis of plants before extraction

The leaves and small stems of the plant were dried in the shade, at ambient temperature not exceeding 40 °C for 72 h. They were then reduced in powders by crushing in a mixer. The powders coming from various harvests were then mixed in a planetary mixer (Colette) to obtain 3 kg of leaves powder. The granulometry of the powder was analysed using Mastersizer® 2000 (Malvern Instrument, United Kingdom) and the water contents were determined by a thermo gravity balance: Mettler-Toledo HB43-S (Greifensee, Switzerland).

2.2.2. Preparation of dry hydro alcoholic extracts

Portions of 35.12 g of dried powdered leaves were macerated for 1 h with 500 ml of ethanol 96%. The unit is agitated mechanically during extraction and all the system is kept at ambient temperature (approximately 30 °C). The mixture is then filtered under vacuum. Filtrate was evaporated to dryness under reduced pressure. The extract is kept in a cold room (6 °C).

For quantification, 100 mg of dried crude extract was dissolved in 10 ml of ethanol. The mixture was then submitted to ultrasounds twice during 15 mn and 1 ml of this solution is taken for the prederivatization.

2.2.3. Prederivatization

Before HPLC injection, the derivatization reaction illustrated in Fig. 1 was applied to each sample to obtain the Q260 compound which can be detected at 260 nm.

One ml of sample (extract solution or standard solution) was transferred into a 10 ml measuring flask. Four ml of 0.2% (m/v) NaOH solution was added in the flask, and then left to react at 50 °C for 30 min. After cooling during 10 min, 1 ml of ethanol was added. Finally the flask was filled with acetic acid 0.2 N.

Table 1
HPLC–UV methods in literature using artemisinin prederivatization for its detection in *Artemisia annua*.

No.	Extraction method	Column	Mobile phase	Derivatization product and RT	References
1	Soxhlet extraction with petroleum ether	Lichrosorb RP18 (10 μm)	Methanol/phosphate buffer (0.01 M; pH 7.9 (55:45)	Q 260 at 16 min	Zhao and Zeng, 1985 [6]
2	Ultrasons extraction with toluene	Waters/Nova-Pak C18 (3.9 × 150, 4 μm)	Acetonitrile/phosphate buffer 10 mm (20:80) – internal standard = acetophenone	Q 260 – RT not indicated	Marchese et al., 2001 [19]
3	Soxhlet extraction with petroleum ether	Discovery C18 SiO_2 (4.6 × 250, 5 μm)	Methanol/acetonitrile/phosphate buffer pH 7.76 (45:10:45)	Q 260 at 17 min	Qian et al., 2005 [17]
4	Maceration with n-hexane	ACE-5 C18 (4.6 × 250, 5 μm)	Formic acid 0.2% (v/v)/acetonitrile (50:50)	Q 260 at 6 min	Erdemoglu et al., 2007 [20]
5	Soxhlet extraction with petroleum ether	Inertsil G8 ODS-3V	Methanol/phosphate buffer 0.01 M (45:55) – internal standard = acetophenone	Q 260 at 12 min	Tonk et al., 2007 [21]
6	Maceration with petroleum ether	Lichrospher C18 SiO2 (4.6 × 250, 5 μm)	Methanol/phosphate buffer 0.01 M (50:50)	Q 260 at 20 min	Zhang et al., 2009 [22]

RT: retention time.

All solutions were filtered on a PTFE 0.45 μm membrane before HPLC analysis.

This procedure was applied to all samples.

2.2.4. HPLC conditions

The HPLC apparatus used for the validation analysis was an Hitachi Alliance from VWR with LaChrom Elite software for data acquisition.

Chromatographic separation was performed with a reversed phase RP-18 LiChroCART column (250 mm × 4 mm I.D.; particle size: 5 μm). Mobile phase consisted in a mixture of methanol and phosphate buffer (5.0 mM; pH: 6.3) (45/55, v/v). A flow rate of 1 ml/min and detection at 260 nm were used. The column was maintained at 35 °C and the injection volume was of 20 μl.

2.2.5. Validation experimental design

In order to validate the analytical method, two kinds of samples were prepared in an independent way: calibration standards and validation standards. The calibration standards are samples without matrix, containing known concentrations of the analyte of interest and are only used for calibration.

The validation standards are reconstituted samples within the matrix containing known concentration of the analyte of interest which are considered as true values by consensus.

As *A. annua* is a biological matrix, relatively large acceptance limits are prescribed [26]. The acceptance limits were thus settled at ±20%. Validation of the method was done for 4 days by testing the following criteria: response function, linearity, trueness, precision (repeatability and intermediate precision), accuracy, limits of detection (LOD) and quantification (LOQ), and quantification range. The accuracy profile methodology for method validation was applied [23,24].

2.2.5.1. Calibration standards. Stock solutions of artemisinin (calibration stock solutions) were prepared in ethanol at 1 mg/ml and stored at 0 °C. Calibration stock solutions and calibration standards were newly prepared for each experiment. Four concentrations (m_{cal} = 4) were used by introducing respectively 0.1, 0.2, 0.4 and 0.6 ml of calibration stock solution into 10 ml measuring flask before derivatization as described above (Section 2.2.3). The final concentration levels of the calibration standards ranged from 10.0 to 60.0 μg/ml. Each concentration was analysed three times (n_{cal} = 3) for 4 days (k_{cal} = 4).

2.2.5.2. Validation standards. For validation standards, a validation stock solution containing an extract solution (≈0.2 mg/ml in artemisinin) was first prepared; validation standards were obtained from 10 ml of this solution spiked with pure artemisinin (8.8 mg) and sonicated for 15 min for complete dissolution and stored at 0 °C.

Validation stock solution and validation standards were also newly prepared for each experiment. Four concentrations were finally reached as described for the calibration standards and ranging from 10.0 to 54.0 μg/ml. Each validation standard was analysed in triplicate (n_{val} = 3) for 4 days (k_{val} = 4).

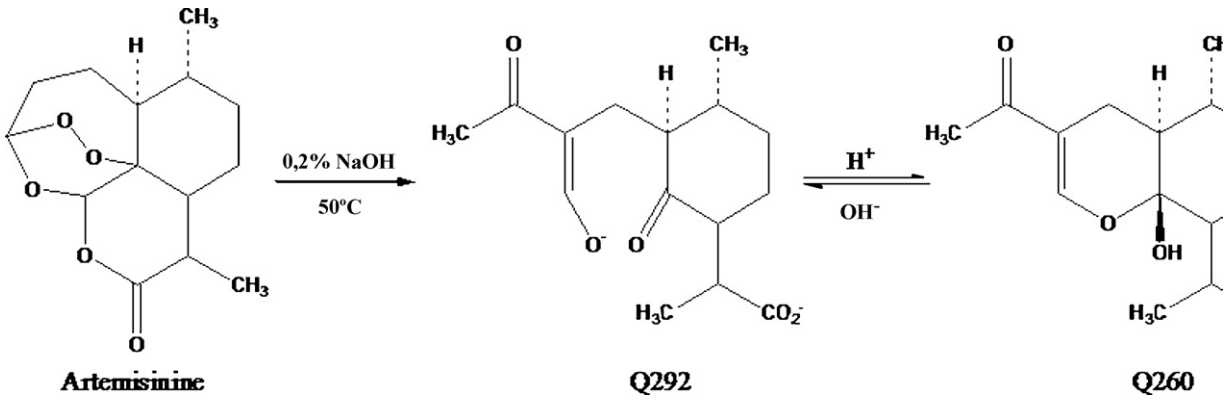

Fig. 1. Derivatization of artemisinin into Q260 compound.

2.2.6. Evaluation of the extraction rate

To evaluate the saturability of the extraction, the extraction procedure was repeated after addition of 10, 20 and 30 mg pure artemisinin to the plant powder. Each extraction was repeated 3 times (a total of 12 extractions, including the triplicate analysis of the samples without any added artemisinin). Artemisinin contents of these extracts were determined as indicated in Sections 2.2.2 and 2.2.3.

2.2.7. Computations

Statistical analyses of the validation data were done using the e-noval V3.0 (Arlenda, Liège, Belgium) software whereas the results of method transfer were processed using Transval V1.0 (Arlenda).

3. Results and discussions

3.1. Extraction procedure

Fig. 2 shows that no saturation is observed for the hydro alcoholic extractions of *A. annua* at the tested concentrations. In fact, whatever the quantity of artemisinin spiked in the conditions of the study, we can observe a proportional increase of the HPLC Area under Curve (AUC). It also proves that the derivatization reaction is complete whatever the artemisinin quantity in this extract, in the experimental range.

The extraction recovery obtain from this experiment is 102.35 ± 16.76%. We can note that it is a good recovery.

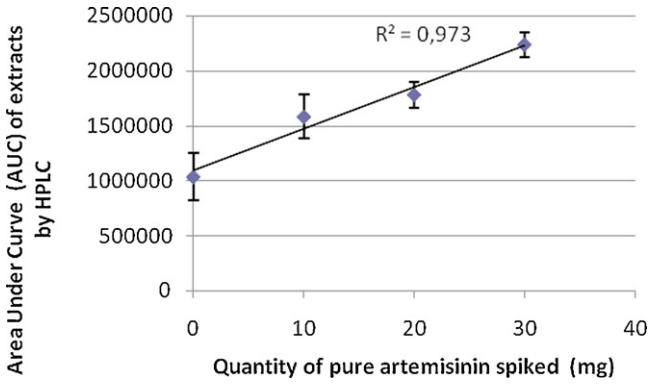

Fig. 2. Artemisinin responses in spiked hydro alcoholic extracts of *Artemisia annua L.* ($n = 3$ for each extract).

3.2. Method validation

3.2.1. Method selectivity

Fig. 3 shows the chromatogram of a standard solution spiked at a concentration of 60.0 μg/ml of artemisinin after derivatization. This figure shows that the developed method allows a rapid separation and detection of Q260 with a retention time (RT) of 10.5 min. The unknown impurity in standard solutions observed at RT of 4 min was also found by others authors [19–22].

Fig. 4A and B shows chromatograms of hydro alcoholic extract. In Fig. 4A no derivatization was done. It can be observed on

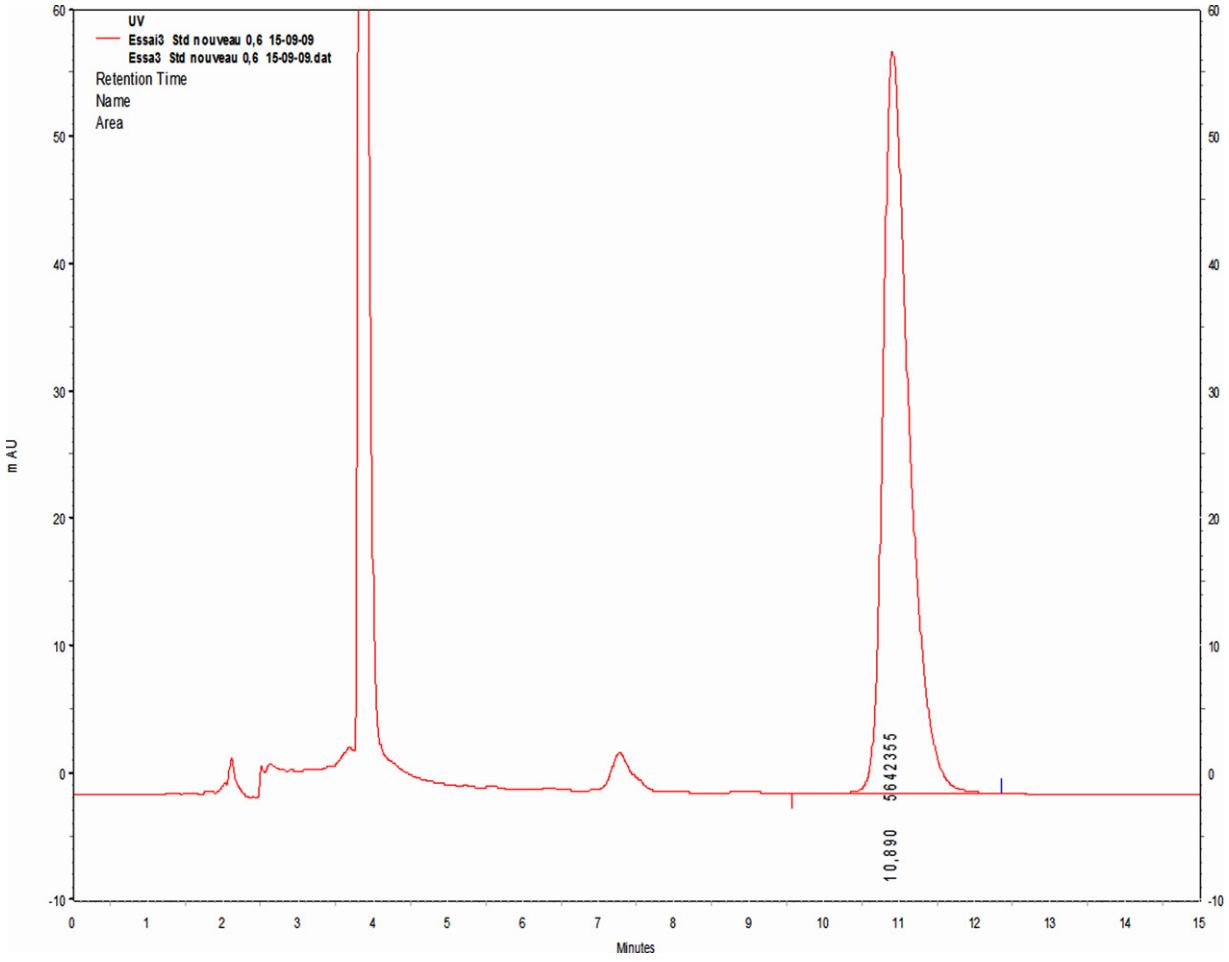

Fig. 3. Chromatogram of a 60.0 μg/ml standard solution of artemisinin derivatized and detected at 260 nm.

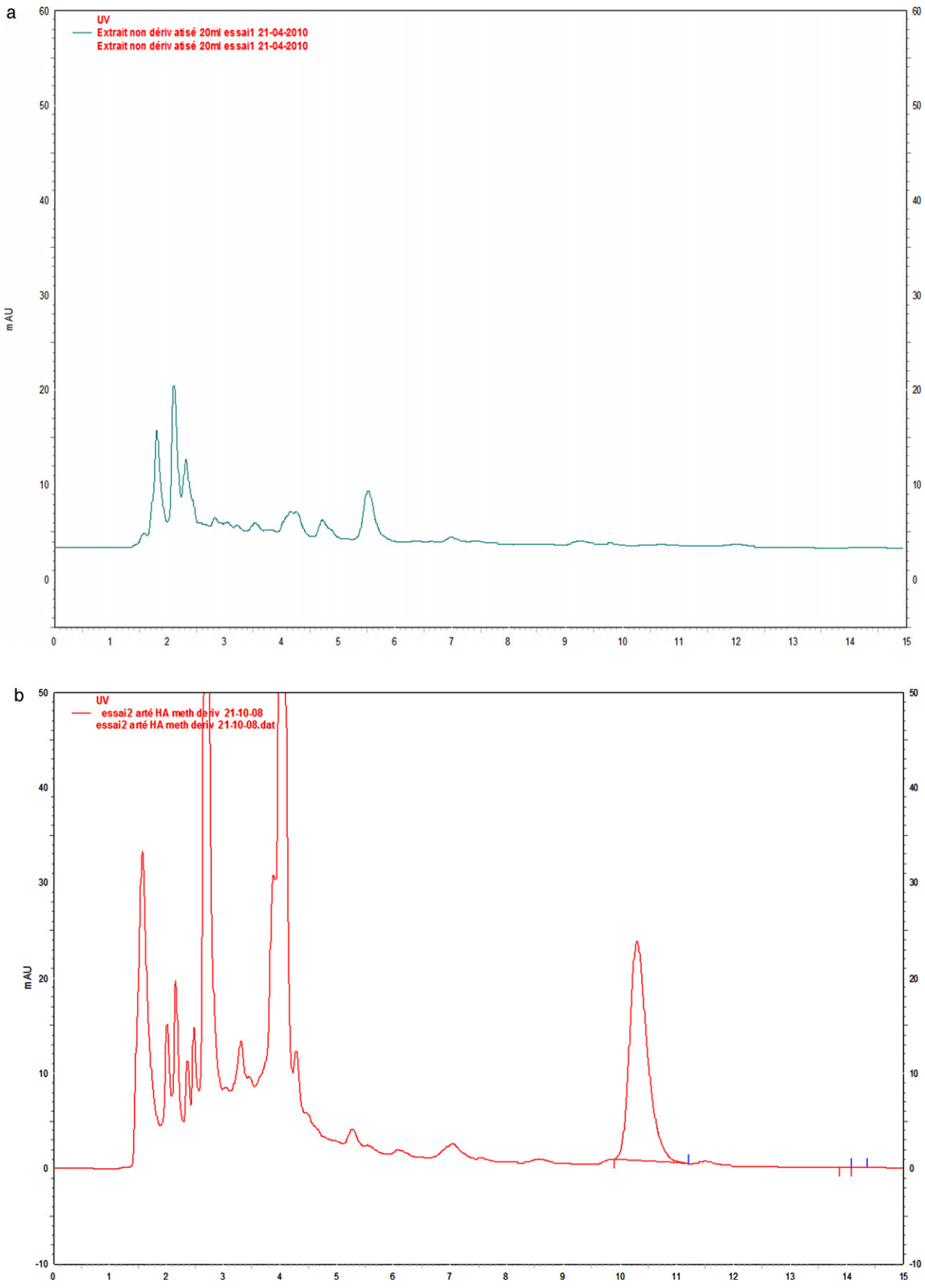

Fig. 4. (A) Chromatogram of a sample of hydro alcoholic extract underivatized and detected at 260 nm. (B) Chromatogram of a sample of hydro alcoholic extract derivatized and detected at 260 nm.

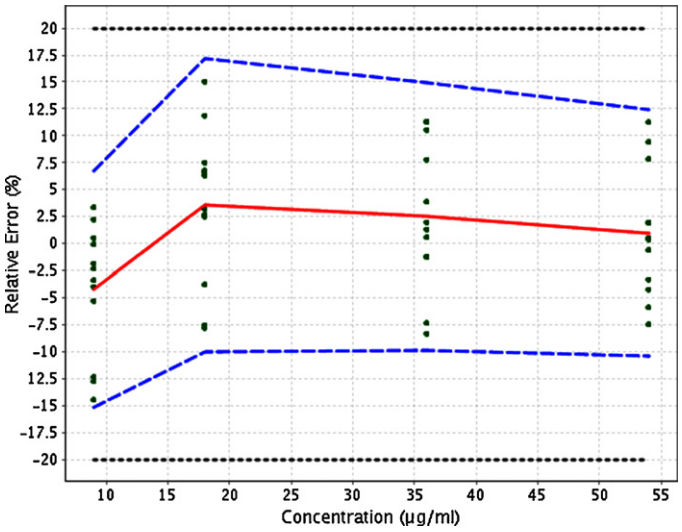

Fig. 5. Accuracy profile obtained by considering the linear regression forced through the origin and fitted with the highest calibration concentration level (60.0 µg/ml). Continuous line: relative bias; dotted lines: ±20% acceptance limits; dashed lines: 80% β-expectation tolerance limits; dots: relative back-calculated concentrations of the validation standards.

this figure the absence of Q260 at RT of 10.5 mn. However, in Fig. 4B, obtained after derivatization, the peak of Q260 is effectively present. This illustrates the adequate selectivity of the developed chromatographic procedure.

3.2.2. Quantitative performance

Different regression models were tested and accuracy profiles were then plotted to determine the most suitable regression model [23,24,27]. Fig. 5 shows the accuracy profile obtained with the linear regression forced through 0 and fitted only with the concentration level of 60.0 µg/ml as response function. It was selected as the most adequate one as this regression model was the sole having the 80% β-expectation tolerance intervals totally included inside the ±20% acceptance limits for each concentration level of the validation standards. It has to be noted that it is not uncommon that a single point calibration curve is used to allow accurate quantification of analytes in complex matrices [28].

Trueness [23,24,27,29] is expressed in relative bias (%) at each concentration level of the validation standards. Relative bias was less than 4.2% (Table 2) showing the excellent trueness of the method.

Precision was evaluated in terms of relative standard deviation (RSD %) values for repeatability and intermediate precision [29]. As seen in Table 2, RSD (%) for repeatability and intermediate precision did not exceed 8%.

Results accuracy implies evaluating total error, the sum of systematic and random errors of the analytical procedure [23,24,27]. As illustrated in Fig. 5, the accuracy profile shows that the relative upper and lower 80% β-expectation tolerance limits are totally included inside the acceptance limits set at ±20%. The method can thus be considered as accurate between 10.0 and 54.0 µg/ml. Indeed, there is at least 80% probability that each future result obtained by the analytical method will be within the ±20% acceptance limits.

The LOD was estimated at 2.73 µg/ml using the mean intercept of the calibration model and the residual variance of the regression. The LOQ was determined with the accuracy profile as the smallest concentration levels where the 80% β-expectation tolerance limits remain inside the ±20% acceptance limits [23,24,27,29]. In other words, this is the smallest concentration level with a maximum total error of 20%. As shown in Fig. 5, the LOQ is the smallest concentration level of the validation standards: 10.0 µg/ml.

Table 2
Validation results for artemisinin quantification in hydro alcoholic extracts of *Artemisia annua*.

Validation criteria	Artemisinin	
Response function	Linear regression through 0 fitted with the level 60.0 only	
Trueness		
Concentration (µg/ml)	Relative bias (%)	
10.0	−4.2	
18.0	3.6	
36.0	2.5	
54.0	1.0	
Concentration (µg/ml)	Repeatability (RSD %)	Intermediate precision (RSD%)
10.0	2.9	6.5
18.0	2.4	7.7
36.0	3.2	7.3
54.0	2.2	6.5
Accuracy		
Concentration (µg/ml)	80% β-expectation tolerance interval (%)	
10.0	[−15.1,6.7]	
18.0	[−10.0,17.2]	
36.0	[−9.8,14.9]	
54.0	[−10.4,12.4]	
Linearity		
Slope	1.017	
Intercept	−0.5266	
r^2	0.9862	

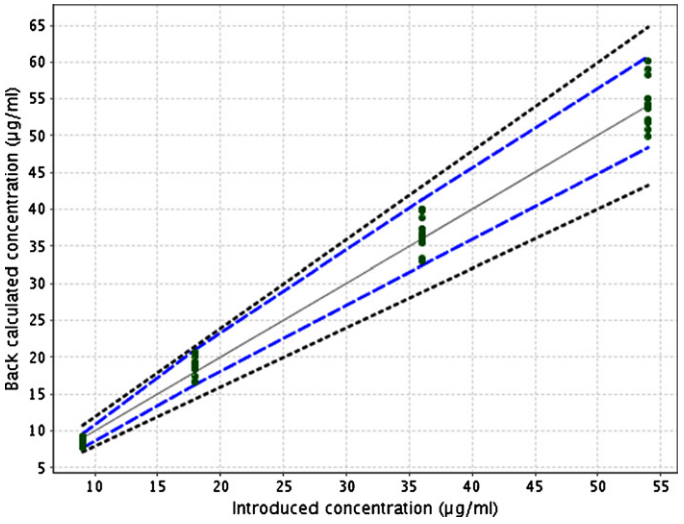

Fig. 6. Linear profiles of artemisinin in hydro alcoholic extracts. The continuous line is the identity line ($y = x$), the dotted lines are the upper and lower ±20% acceptance limits expressed in concentration values and the dashed lines are the upper and lower β-expectation tolerance limits (β = 80%) also expressed in concentration units.

Results linearity demonstrates the relationship between introduced and calculated concentration [23,24,27,29] and is assessed using the β-expectation tolerance interval approach. In order to demonstrate the linearity of the results obtained by the analytical method, a regression line was fitted on the calculated concentrations of the validation standards as a function of the introduced concentrations by applying a linear regression model. The equation obtained with the coefficient of determination is presented in Table 2. The slope and intercepts values are close to 1 and 0, respectively indicating the absence of proportional and constant systematic error. Fig. 6 demonstrates the linearity of the results by showing that the 80% β-expectation tolerance intervals are

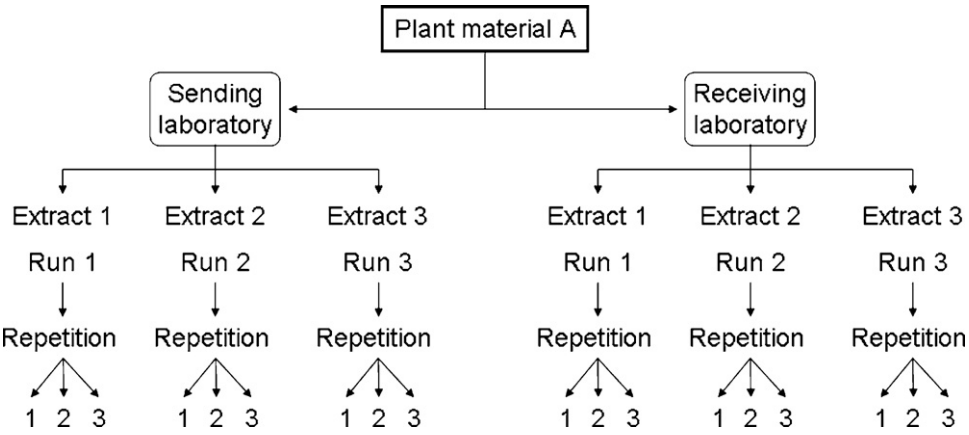

Fig. 7. Experimental design used to evaluate the transferability of the HPLC–UV method for the quantification of artemisinin from the R&D laboratory to the routine laboratory.

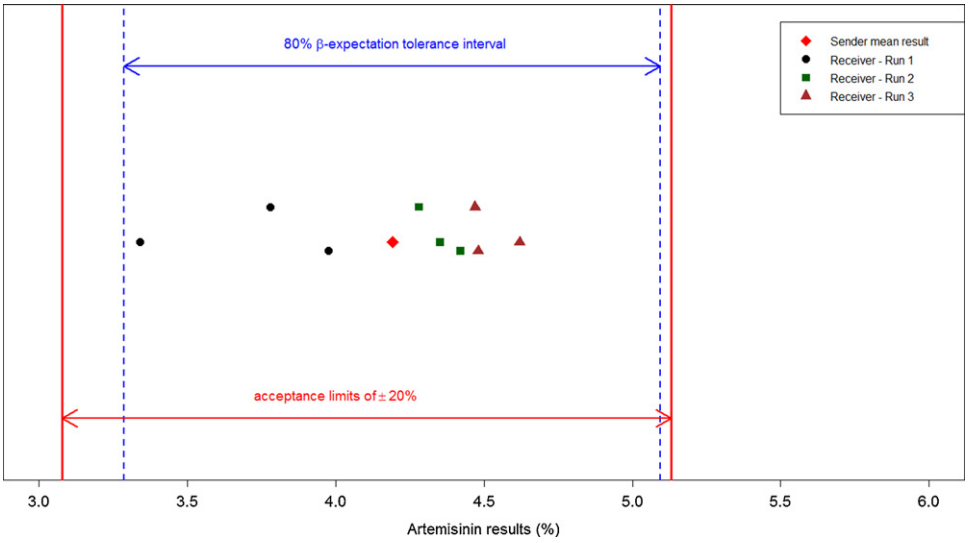

Fig. 8. Decision profile of the transfer of the HPLC–UV method for the quantification of artemisinin from the R&D laboratory (sender) to the routine laboratory (receiver).

included into the acceptance limits at each concentration level of the validation standards.

3.3. Inter-laboratories transfer of the HPLC–UV method

The developed and validated analytical method is aimed to be used in routine experiments not in the initial research and development laboratory (or sending laboratory) but in a routine laboratory based in Benin (called receiving laboratory). Therefore this analytical method had to be transferred to this laboratory. However, the sole transfer of the documented analytical procedure is not sufficient to ensure that results that will be generated at the receiving laboratory will be of adequate quality and reliability. Therefore, the experimental demonstration that the receiving laboratory will provide in future routine analyses results that are close enough to those that would have been generated by the sending laboratory should be performed. To achieve this, an inter-laboratories transfer study was performed following the design illustrated in Fig. 7.

As shown in Fig. 7, three hydro alcoholic extracts of *A. annua* were prepared with the same plant material (method described in Section 2.2.2) in each of the two different laboratories to assess the transferability of the HPLC method. The operators and the equipments were different in each laboratory involved in the method transfer. The analysis of each extract was repeated three times and the transfer involved three different runs (one run per extract) in each laboratory as shown in Fig. 7. The artemisinin content of the extracts were therefore determined in each laboratory by different HPLC equipments, different days and different operators. To assess the transferability of the HPLC method, a tolerance interval approach was implemented [25,30]. To be coherent with the requirements used for the method validation at the sending laboratory, the minimum probability to obtain results at the receiving laboratory within acceptance limits of ±20% was set at $\beta = 80\%$. This means that it is accepted that each future result of the receiving laboratory must have at least 80% chance to be around ±20% of the reference result provided by the sending laboratory.

The results obtained for the method transfer are depicted in Fig. 8.

It can be seen from Fig. 8 that the receiving laboratory slightly systematically over-estimates the results obtained by the sending laboratory by about 2%. Fig. 8 also indicates that each future result that will be obtained by the receiving laboratory will have 80 chances out of 100 to be between 3.3% and 5.1% of artemisinin content. Fig. 8 shows that the transfer is perfectly acceptable as the 80% β-expectation tolerance interval of the receiver results is fully included inside the ±20% acceptance limit around the mean result of the sending laboratory. Hence the reliability of the results that will be routinely obtained by the receiving laboratory is guaranteed.

Table 3
Estimates of the measurement uncertainties related to artemisinin in hydro alcoholic extracts at each concentration level investigated in validation using linear regression through 0 fitted with the maximum calibration level only.

Concentration level (μg/ml)	Uncertainty of the bias (μg/ml)	Uncertainty (μg/ml)	Expanded uncertainty (μg/ml)	Relative expanded uncertainty (%)
9.0	0.2703	0.6414	1.283	14.25
18.0	0.6728	1.546	3.093	17.18
36.0	1.224	2.892	5.785	16.07
54.0	1.695	3.912	7.823	14.49

Table 4
Estimates of the measurement uncertainties related to artemisinin in hydro alcoholic extracts obtained with the results of both laboratories during the method transfer assessment.

Uncertainty of the bias (%)	Uncertainty (%)	Expanded uncertainty (%)	Relative expanded uncertainty (%)
1.7	3.95	7.9	19.0

3.4. Measurement uncertainty

Demonstrating the reliability of analytical results is essential in order to interpret them correctly and method validation is only a first step to achieve this. Indeed, it is not enough if one aims at interpreting and comparing results correctly. Uncertainty of measurements must therefore be evaluated to ensure this [31]. Uncertainty of the measurement characterises the dispersion of the values that could reasonably be attributed to the measurand [31,32].

3.4.1. Uncertainty obtained from the method validation

The accuracy profile validation methodology allows, without any additional experiments, to obtain estimation of measurement uncertainty [33]. Several estimations of uncertainty were computed and are presented in Table 3. The expanded uncertainty was computed using a coverage factor of $k = 2$ [31,32], representing an interval around the results where the unknown true value can be observed with a confidence level of 95%. Table 3 shows that relative expanded uncertainties were less than 18% which means that the unknown true value is located at a maximum of ±18% around the measured result with a confidence of 95%.

3.4.2. Uncertainty obtained from the method transfer

Similarly as done during the method validation realised at the sending laboratory, the measurement uncertainty of the HPLC–UV method of the receiving laboratory was directly estimated from the method transfer study. The expanded uncertainty was computed with a coverage factor of 2, defining a region where the true value can be observed with a confidence level of 95%. Table 4 shows all the uncertainty estimates obtained from the results obtained by both laboratories. In particular, this table shows that the relative expanded uncertainty was of 19% which means that the unknown true value is located at a maximum of ±19% around the measured result.

3.4.3. Measurement uncertainties comparison

The value of relative uncertainty estimated during the method transfer (19%) is extremely close to the maximum 18% value obtained during the validation of the HPLC–UV method. This shows that the uncertainty estimation made during the method validation with spiked validation standard is a good estimation of the uncertainty of real samples. Fig. 9 corroborates this. Indeed this figure shows the linear relationship between the concentration of artemisinin and the uncertainty obtained during the method validation. In addition the 95% prediction interval is depicted. From Fig. 9, it can be seen that the uncertainty obtained during the transfer study (3.95%) is included within this prediction interval confirming the adequacy of the estimation of measurement uncertainty obtained during method validation. Furthermore, the laboratory in which the analyses of artemisinin were obtained was not found to influence the results when modelling the results with a linear mixed model (p-value = 0.7649). This highlights the robustness of the analytical procedure to inevitable differences in its implementation at each laboratory.

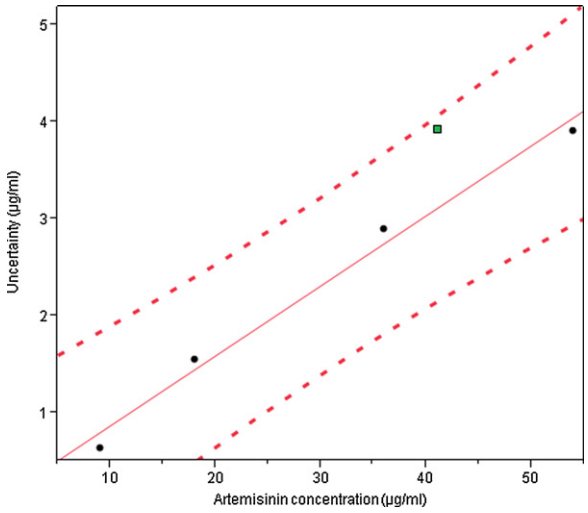

Fig. 9. Measurement uncertainty comparison. The filled circles represent the uncertainty estimated at the sending laboratory during method validation, the continuous line is the simple linear regression of the uncertainty estimated at the sending laboratory during method validation (filled circles) over the concentration of artemisinin: $y = 0.0719x + 0.1433$, $r^2 = 0.9884$. The two dashed lines delimit the 95% prediction interval of the linear regression. The single filled rectangle is the measurement uncertainty estimated during the method transfer study.

4. Conclusion

The hydro alcoholic extracts of *A. annua* were selected for therapeutic formulations of antipaludic drugs. In fact, this extract allows the extraction of the majority of the plant's artemisinin and it also avoids the problems of solvent elimination since ethanol is not toxic. Determination of artemisinin in this kind of extracts is not easy particularly in countries like Benin where equipments in laboratories are limited.

The development of this HPLC–UV method is intended to contribute to standardization of the hydro alcoholic extraction procedures, which is essential to develop drugs based on crude extracts of plants. This assay proved to be selective and was fully validated for the quantification of artemisinin in hydro alcoholic extracts of *A. annua* after derivatization. The method developed and validated was then successfully transferred to a laboratory in Benin by showing that the quality of the results that it will generate during routine application of the method is sufficient. Finally, the measure-

ment uncertainty of the method was estimated from the validation experiments on spiked samples as well as from the transfer study on authentic unspiked samples. The comparison of these measurement uncertainty estimations showed that they were coherent. Thus estimation of measurement uncertainty from a validation experiments predicts well the measurement uncertainty of real routine samples.

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