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Spectrophotometric determination of antiplasmodial cochloxanthins from roots of *Cochlospermum planchonii* Hook.f. (Bixaceae)

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ABSTRACT

The roots and leaves of *Cochlospermum planchonii* and *C. tinctorium* are widely used in Burkina Faso as medication for malaria treatment. The cochloxanthins, carotenoids, are - at least partially - responsible for the antimalarial activity of *Cochlospermum* spp.. Because of large variation of cochloxanthin contents in rhizomes and leaves of *Cochlospermum* spp., the available products are not always reliable. The gold standard method for the quantification of cochloxanthins is HPLC. However, HPLC is expensive, time consuming, and needs experienced personnel. Therefore, for quick analysis of *Cochlospermum* roots in local pharmacies and companies, we developed a spectrophotometric method that is inexpensive, fast, simple and robust. In our study, we produced ethanolic extracts of market samples of roots of *Cochlospermum* sp. from Burkina Faso and analysed them in parallel by HPLC and spectrophotometry. The two methods correlated well with an $R^2 > 0.95$ ($P < 0.001$). However, spectrophotometry overestimated the samples by 10% compared to HPLC analyses. For the spectrophotometric method, the coefficients of variation inter day and intra days were 9% and 12.8%, respectively with a limit of detection (LOD) of 0.65 µg/g. A parallel test of the spectrophotometric assay in a 96 well microplate format showed a good correlation ($R^2 = 0.96$) with conventional spectrophotometry. Variation of sample volumes in the wells did not influence the results, but extreme values were not correctly measured in the microplate reader. The new spectrophotometric method is well suitable for the analysis

Abbreviations: ACT, artemisinin-based combination therapy; CV, coefficient of variation; DAD, diode array detection; LOD, limit of detection; LOQ, limit of quantification.

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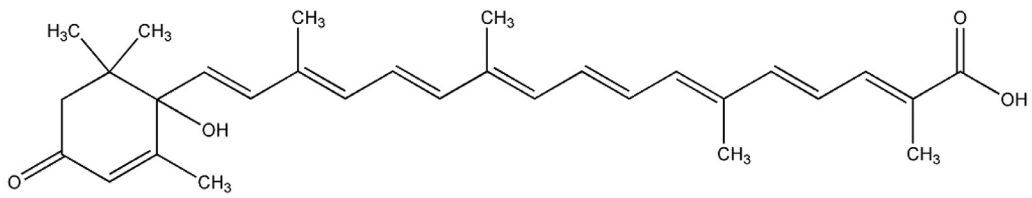
of cochloxanthins in *Cochlospermum* spp. roots; however, due to interfering absorbance, plant material containing chlorophyll cannot be analysed.

Introduction

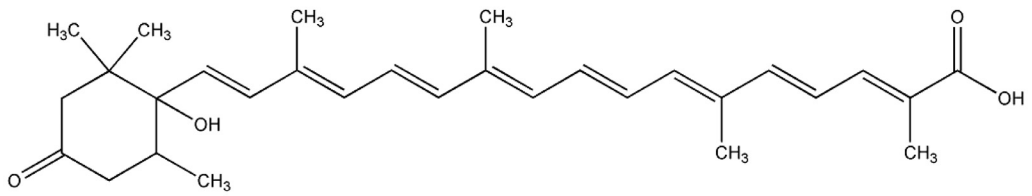
Natural products always played an important role in human medicine, and still constitute more than 30% of the pharmaceutical market today [17]. Malaria is an infectious disease caused by *Plasmodium* spp [22]. Currently, artemisinin-based combination therapy (ACT) is the treatment of choice to cure uncomplicated *P. falciparum* malaria [20]. Apart from chloroquine resistance in Africa, *P. falciparum* also showed resistance against artemisinin in other regions of the world [15,19]. It is of great importance to find alternative treatments and develop new drugs to replace the ones that become ineffective [6]. Moreover, new therapies should be inexpensive and easy to handle [10]. The search for new drugs from nature, also known as ethnopharmacology plays a huge role in this topic, as malaria is mainly treated with natural products [13]. Ethnopharmacological investigations in Burkina Faso alone identified 72 plant species being used in traditional medicine to treat malaria [11].

In West Africa, two species of the genus *Cochlospermum, C. planchonii* and *C. tinctorium* are used against malaria [21]. In Burkina Faso, *Cochlospermum* species are known as "N'Dribala" and are sold in local markets or pharmacies, as dried rhizomes or as tea or in capsules [23]. Studies with *Cochlospermum* spp. extracts showed anti-plasmodial activity [18]. In *C. tinctorium*, the highest anti-plasmodial activity was found in the root extracts [4]. Decoctions of *C. planchonii*, tested on patients with uncomplicated *P. falciparum* infections demonstrated their efficacy against malaria compared to the standard chloroquine medication [3]. The roots of *C. planchonii* showed enhanced anti-plasmodial activity when extracted with ethanol, as compared to the decoction [2,12]. Further extraction of the roots of *Cochlospermum* spp. with organic solvents resulted in the characterisation of 5 major, active compounds including the carotenoids cochloxanthin (A) and dihydrocochloxanthin (B) [2]. The cochloxanthins are unique for the genus and present in both species in a similar composition, with a ratio of (A) to (B) at roughly 1:2. While the combined content of (A) and (B) makes up more than 70% of all cochloxanthins, the sum of the other 10 derivatives remains below 30% [12] (Scheme 1).

Saye tea is a mixture of roots of *C. planchonii*, leaves of *Phyllanthus amarus* and *Cassia alata* [7]. *C. planchonii* is mainly collected from the wild; however, domestication started recently [8]. Apart from the risk of possible extinction of *Cochlospermum* spp., harvesting from the wild leads to high variations in the cochloxanthin content, as the rhizomes from wild populations are never uniform in their content of secondary metabolites. Therefore, it is important to determine the quantity of cochloxanthins in plant material.



(A)



(B)

Scheme 1. Cochloxanthin (A), dihydrocochloxanthin (B) [1].

The cochloxanthins in *Cochlospermum* spp. have been isolated and analysed by HPLC/UV and characterised by NMR [12]. For routine analysis of plant material, expensive apparatus and time-consuming analyses are not suitable. An inexpensive, robust and quick method would be of great interest. As cochloxanthins are yellow to orange in colour, a spectrophotometric method could enable a routine testing of incoming raw materials. Spectrophotometric and HPLC methods for the determination of carotenoids in food have been compared favourably [5,9,14]. Here, we show that a simple, fast, and robust spectrophotometric method can be used for routine determination of cochloxanthin concentrations in the roots of *Cochlospermum* spp.

Material and methods

Plant material

Samples of roots of *C. planchonii* were collected from different vendors of the markets of Bobo-Dioulasso and Ouagadougou. The phytomedicines of N'Dribala, consisting only of roots of *Cochlospermum planchonii* were provided by Phytofla (Banfora, Burkina Faso). Reference samples were collected from the wild in South-West Burkina Faso and herbarium specimens of both *C. planchonii* (ID 16712) and *C. tinctorium* (ID 16711) were deposited at the herbarium of the University of Ouagadougou.

Standard

Curcumin (CAS-No.: 458-37-7; Carl Roth, Karlsruhe, Germany) was chosen as standard for spectrophotometry as well as HPLC analysis due to a similar absorbance maximum and solubility in ethanol when compared to cochloxanthins. Starting with a 1 molar (368.39 mg/ml) concentration of curcumin in ethanol (100%), dilutions of 18.4, 9.2, 4.6, 2.3, and 1.2 µg/ml were made to serve as standards to generate a standard curve for concentration measurements.

Extraction

For the extraction, dried rhizomes of *C. planchonii* and *C. tinctorium* were ground in a mill (MM301, Retsch, Haan, Germany). Of all 25 samples, 0.5 g of plant material was used and extracted with 10 ml of ethanol (100%) (Chem-Lab, Zedelchem, Belgium) for one hour in an ultrasonic bath at 23 °C. Ethanol was chosen due to the highest possible solubility of cochloxanthins [12]. The extracts were filtered (Whatman Grade 604 Qualitative Filtration Paper, Sigma-Aldrich, Vienna, Austria) and kept in dark vials in the refrigerator at 4 °C until further analysis.

Quantification of cochloxanthins by HPLC

The cochloxanthins were determined using the method of Lamien-Meda et al. [12] with the modification of using curcumin instead of cochloxanthin as a calibration standard for HPLC as well as for spectrophotometry. The HPLC system used was from Shimadzu (Korneuburg, Austria) consisting of the CTO-20AC column oven (23 °C), SIL-20AXR autosampler, Nexera XR LC-20ADXR pump station, DGU-20A5R degasser and CBM-20A controller DAD-detector. The system was equipped with a C18 Atlantis® 4.6 × 150 mm, 5 µm column (Waters, Milford, MA, USA) and operated at 23 °C. The mobile phase used was 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) at a flow rate of 1 ml/min with an elution program as follows: 0–4 min 10% B, 4–10 min linear gradient 10–90% B, 10–15 min 90% B, 15–16 min linear gradient 90–100% B, 16–21 min 100% B. 21–22 min decreasing gradient 100% to 50% B, followed by 22–27 min 50% B, 27–28 min decreasing gradient 50–10% B, 28–35 min 10% B. The injection volume was 10 µl and data was analysed at 435 nm.

Upon separation of cochloxanthins, up to 12 peaks could be identified (Fig. 1). Each peak was evaluated by checking the already known absorbance spectrum of cochloxanthins. Cochloxanthin (A) and dihydrocochloxanthin (B) were the main compounds (Fig. 2). The concentration of total cochloxanthins was determined by adding up all cochloxanthin values.

The validation of the HPLC method was done as described by [16].

Spectrophotometry

Spectrophotometry was performed by using a V-550 UV-VIS spectrophotometer (JASCO, Pfungstadt, Germany) at a wavelength of 415 nm and 23 °C. Curcumin was used to prepare a calibration curve (concentration range 1.2 – 36.8 µg/mL, $R^2 > 0.99$). The extracts were diluted with ethanol (100%) in a ratio of 1:10 to fit into a suitable absorbance range. The amount of cochloxanthins in the extracts were expressed as µg curcumin equivalent (CE) / g plant material. To make sure that the extracts only contain *Cochlospermum* spp. rhizomes, an absorbance spectrum should be determined. The measured extracts showed the typical cochloxanthin peaks at around 436 and 461 nm (Fig. 3), as described previously [12].

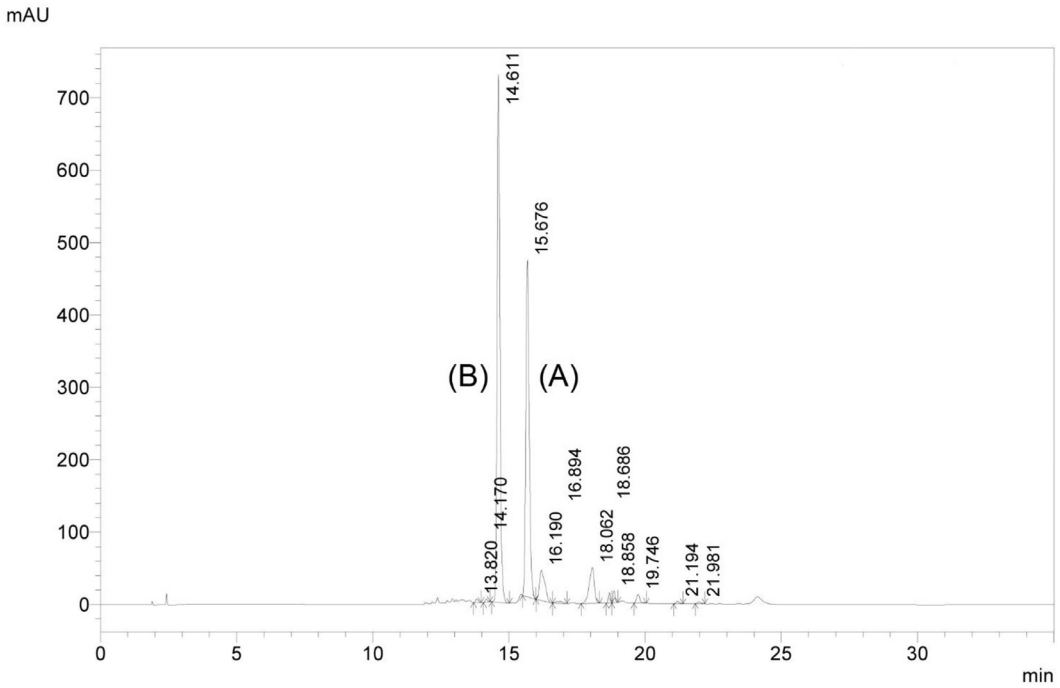


Fig. 1. HPLC chromatogram of *Cochlospermum planchonii* market sample 16.

Validation

Using the UV/VIS spectrophotometer, six samples were randomly chosen and extraction and analyses were repeated on four days to verify the repeatability of the method. One randomly chosen sample was used to verify the reproducibility of the method. Extractions and analyses were done as described in the paragraphs "extraction" and "spectrophotometry" and repeated 8 times with the same sample on the same day. To evaluate the limit of detection, a randomly chosen sample of known content was extracted, then serially diluted in ethanol (100%).

To test the ruggedness of the method, the identical six samples as mentioned above (including one with a low and one with a high concentration) were measured in parallel on a standard 96-well microplate in a Bio-Rad iMark^{TM} Microplate Reader (BioRad, Vienna, Austria) at 415 nm with different sample volumes (50 µl, 100 µl, 150 µl, 200 µl) corresponding to a calculated layer thickness of 1.4 mm, 2.9 mm, 4.3 mm and 5.7 mm, respectively. All measurements have been performed at 23 °C.

Statistical evaluations

All statistical analyses (regression analysis and *t*-test between the two markets) were performed with Microsoft Excel 2016.

Results and discussion

The main intention of this work was to develop an easy and affordable spectrophotometric method to measure the cochloxanthins, antiplasmodial carotenoids in *Cochlospermum* species, in order to evaluate the quality of drug raw materials by determining the contents of cochloxanthins e.g. in pharmacies and producers of herbal medicines in West Africa. In consequence, this method could guarantee a steady quality of *Cochlospermum* spp. supply. Moreover, the method should be suitable for selecting and breeding *Cochlospermum* rhizomes with high contents of cochloxanthins. Domestication and breeding is a necessary step in the near future, because *Cochlospermum* species are declining in nature at a rate, which could lead to extinction [8].

The 'gold standard' for the measurement of carotenoids is HPLC [9]. Therefore, we analysed the *Cochlospermum* spp. extracts by HPLC, as described previously [12]. We used a part of the original, described sample set, consisting of 24 market samples and a reference sample of *C. planchonii*. Unfortunately, the standard of purified cochloxanthins was not stable at −20 °C over the period of one year and therefore we had to use another standard. We decided to use a standard that has a similar absorption maximum to cochloxanthin, that is stable, soluble in ethanol, non-toxic, easily available and inexpensive, criteria fulfilled by curcumin (Supplemental Figure 1 a, b). The HPLC chromatogram (Fig. 1) is comparable to the previously

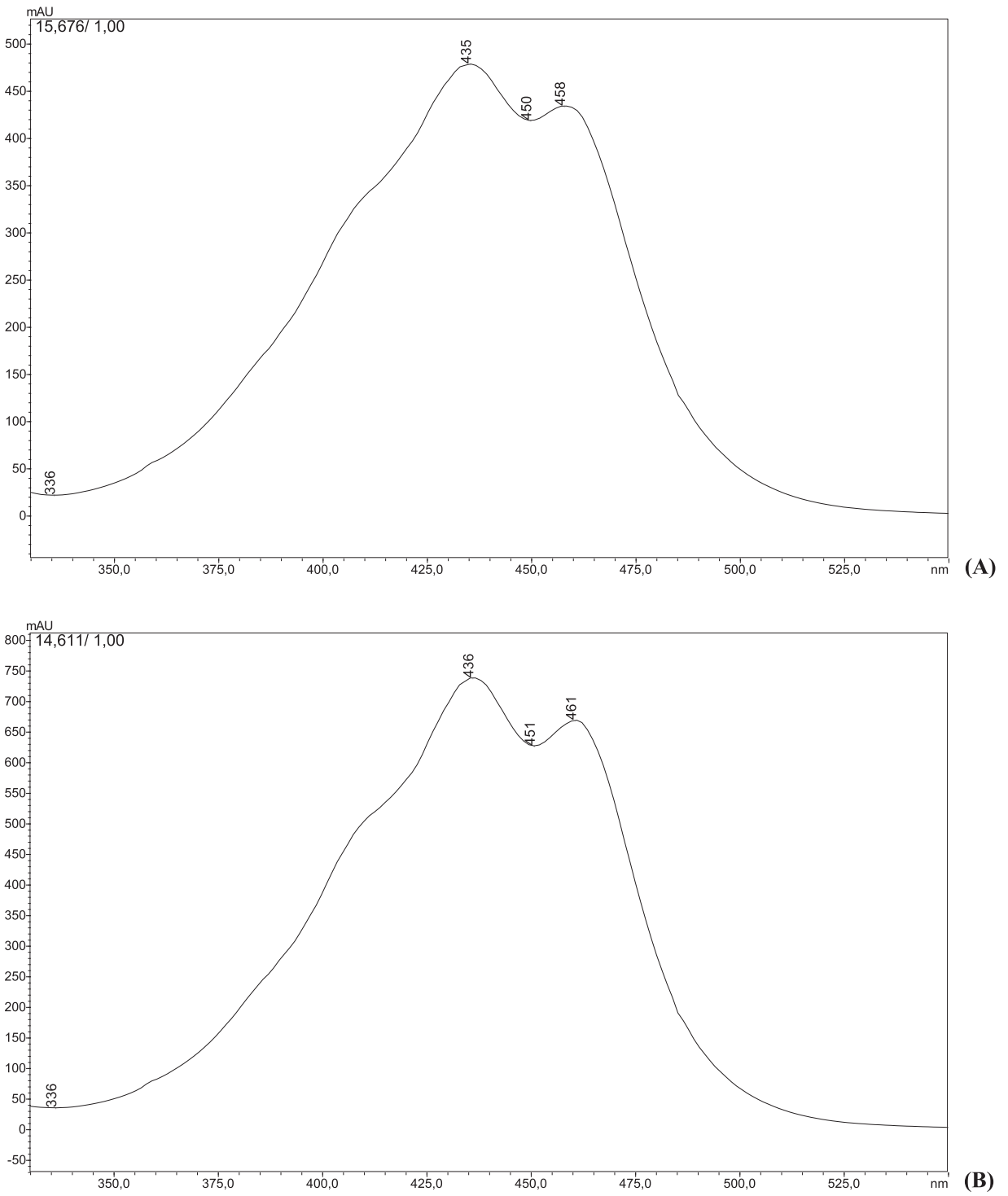


Fig. 2. UV/VIS spectrum of cochloxanthin (A) and dihydrocochloxanthin (B) as measured by the Diode Array Detector.

published results [12], showing the main peaks of cochloxanthine (A) and dihydrocochloxanthin (B). Up to ten additional, minor peaks were identified accordingly, which made up less than 30% of the total content (Supplemental Table 1). Using a diode array detector (DAD), the spectra of eluted compounds were recorded. The spectra of (A) and (B) show the typical carotenoid double peak at 435 and 460 nm (Fig. 2), while minor components showed some deviations in their wavelength maxima and form of spectrum (Supplemental Table 1; Supplemental Figure 2). The quantification of HPLC peak areas was performed in connection with a standard curve (Supplemental Figure 3).

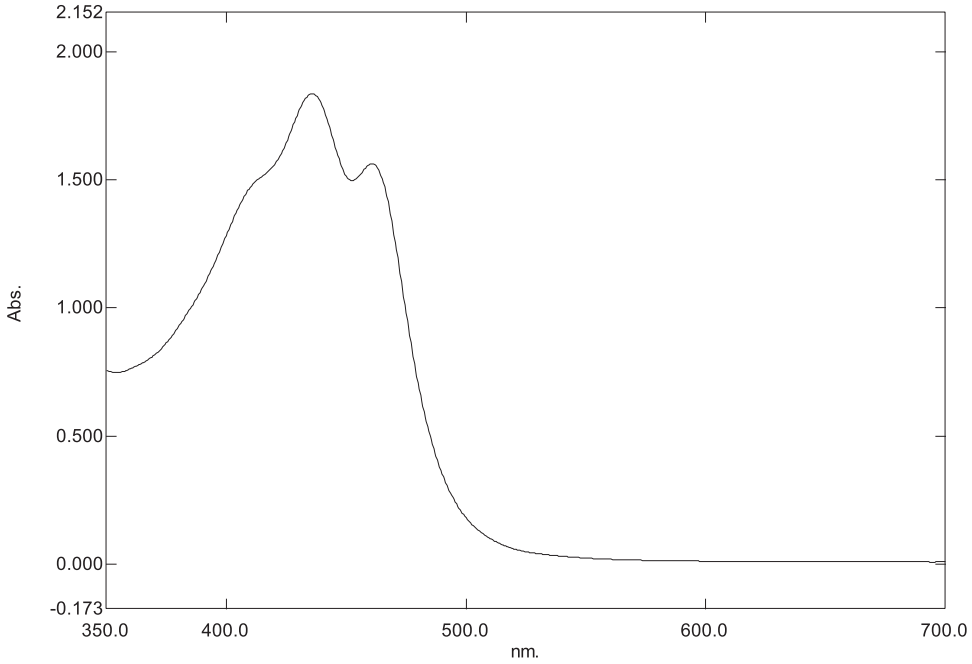


Fig. 3. UV/VIS spectrum of *Cochlospermum planchonii* market sample 16 measured by spectrophotometry.

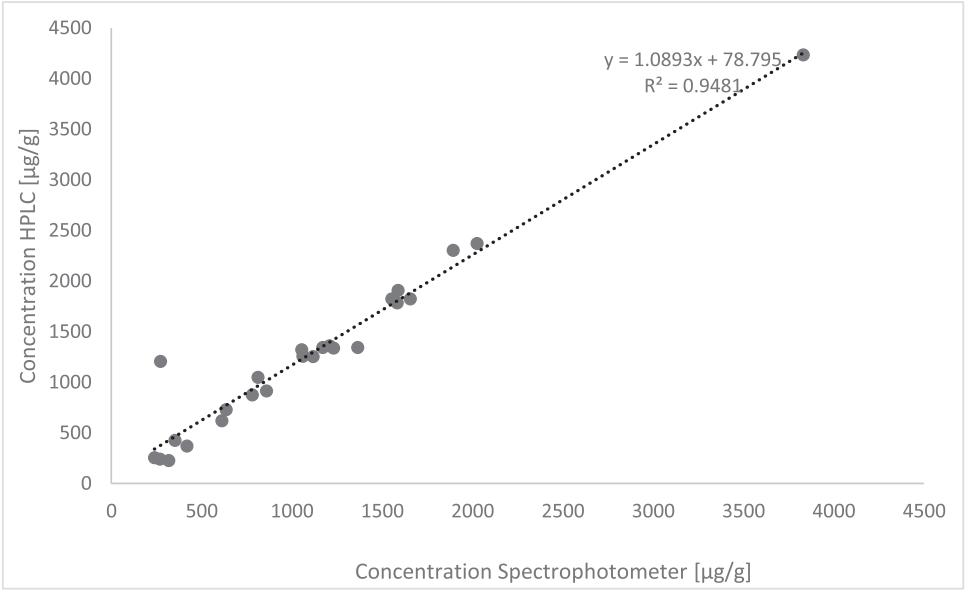


Fig. 4. Correlation of concentrations determined by spectrophotometer and HPLC.

To develop a spectrophotometric method, *Cochlospermum* spp. extracts were measured at the nearest adjustable wavelength to the 435 nm maximum, i.e. 415 nm, and quantified by using a standard curve (Supplemental Figure 4). The same curcumin standard used for HPLC was applied for spectrophotometry. Applying this method to the market samples, it was possible to compare the obtained cochloxanthine concentrations to the HPLC results. The correlation of the spectrophotometric assay to the results from the HPLC measurements was very good with an R^2 =0.95 ($F = 421$; df = 23; $P < 0.001$). However, the spectrophotometric method was overestimating the samples by 10%, compared to HPLC results, after excluding the one obvious outlier (Fig. 4). The spectrophotometric method was characterised by coefficients of variation (cv) of the variability within a day and between days of 9% and 12.8%, respectively. The limit of detection (LOD) was 0.65 µg/g and limit of quantification (LOQ) 6.53 µg/g (Table 1).

Table 1
Validation parameters of the spectrophotometric analysis of cochloxanthins in *Cochlospermum* roots.

Parameter	Value
Variation Within a day (repeatability, cv%)	9%
Variation between-days (reproducibility, cv%)	12.8%
LOD (standard spectrophotometer)	0,12 µg/g
LOQ (standard spectrophotometer)	1,24 µg/g
LOD (Microplate Reader)	0,13 µg/g
LOQ (Microplate Reader)	1,3 µg/g

Table 2
Normal ($n=7$) and trimmed ($n=5$) relative similarity of the results obtained by microplate reader versus spectrophotometer.

	Mean similarity of results obtained by microplate vs. standard spectro- photometer [%] ($n=7$)	Mean similarity of results obtained by microplate vs. standard spectro-photometer [%] after trimming ($n=5$)
Sample volume [µl] (path-length [mm])	mean similarity ± SD	mean similarity ± SD
50 (1.4)	98 ± 26	83 ± 6
100 (2.9)	97 ± 11	101 ± 7
150 (4.3)	86 ± 13	93 ± 6
200 (5.7)	99 ± 12	103 ± 8

SD, standard deviation.

The ruggedness of the method was tested by analysing six samples in parallel to the cuvette-based spectrophotometer in a 96-well microplate spectrophotometer with varying sample volumes in the wells (= changing the path-length of the measuring beam) (Table 2). The path-length in the wells had no significant effect on the result. However, it was found, that the microplate measurements of samples with extreme values deviated strongly from that of a standard spectrophotometer, demonstrating the microplate spectrophotometers' limited measurement range. By eliminating the lowest and highest values, the standard deviation was drastically reduced. Therefore, it is very important to adjust the dilution for the microplate measurements correctly. With this method, the LOD and LOQ have also been determined with 0.13 µg/g and 1.3 µg/g, respectively (Table 2).

The reason for the overestimation of the cochloxanthin concentration by the spectrophotometric method when compared to HPLC could be the exclusion of several yellow coloured substances without spectra similar to cochloxanthins. Another explanation could be differences in the absorbance coefficients of cochloxanthins and curcumin at the wavelength used. A third possibility might be the variation of absorbance due to different solvents and pH. For spectrophotometry, ethanol was used as solvent, while HPLC used a gradient of water/methanol containing 0.1% formic acid. Carotenoids are sensitive to light and higher temperatures and prone to oxidation and therefore often not suitable standards, whereas curcumin is a stable compound. Assumingly, the factors of deviation of absorbance coefficients will be equal for both analytical methods, HPLC and spectrophotometry. Therefore, it is possible to compare the quality of measurements in both instruments. Alternatively, the spectrophotometric method can be used for the direct estimation of cochloxanthine concentrations by dividing the absorbance by the average molar absorption coefficient of carotenoids 135310 L/mol*cm [5]. A similar approach has been used for the calculation of total carotenoid absorbance values and showed almost identical values compared to the actual absorbance values [9].

The market samples showed a very wide variation with a mean of 1295 ± 869 µg/g and a range from 227 to 4233 µg/g, similar to previous results [12]. The samples from the two markets, Bobo-Dioulasso and Ouagadougou, did not differ from each other ($P=0.81$). Overall, the same samples re-analysed in this work had an already approximately 30% lower content of cochloxanthins than reported by [12], who analysed the samples two years before. The samples were stored in the meantime at 23 °C, protected from light.

The spectrophotometric method is a good alternative to evaluate the content of cochloxanthins in the roots of *Cochlospermum* spp. and is useful for quality control of the drug and for further breeding selection due to its easy implementation and low costs. However, the method is currently not applicable for analyses of *Cochlospermum* leaves or in mixtures with chlorophyll-containing plant material like Saye tea (mixture of roots of *C. planchonii*, leaves of *Phyllanthus amarus* and *Cassia alata*).

Conclusion

A spectrophotometric method for the quantification of the anti-malarial carotenoids, the cochloxanthins, in the roots of *Cochlospermum* spp. was developed and compared with HPLC analysis. The correlation of these methods was very good ($R^2=0.95$). In parallel, a spectrophotometric assay in a 96 well microplate format showed a good correlation ($R^2=0.96$) with conventional spectrophotometry. Thus, we present here a simple, inexpensive, rapid and robust method for *Cochlospermum* roots quality control that can be used in local pharmacies and laboratories in West Africa.

Declaration of interest

None.

Acknowledgements

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2019.e00055.

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