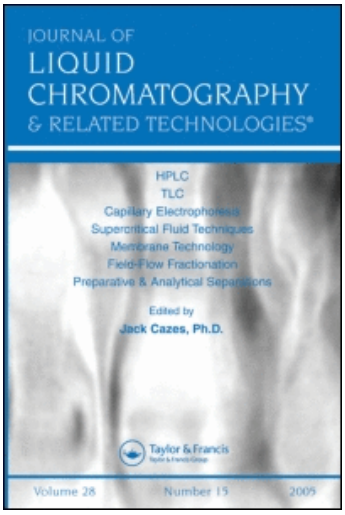

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Quantitative HPTLC Analysis of Artemisinin in Dried *Artemisia annua* L.: A Practical Approach

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Quantitative HPTLC Analysis of Artemisinin in Dried *Artemisia annua* L.: A Practical Approach

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Abstract: Artemisinin, an antimalarial substance, is obtained on a large scale from dried leaves of *Artemisia annua* L. To monitor the plant during growth, determine time of harvest, or evaluate the commercial value of plant material—all with respect to the artemisinin content—it is most important to have available a simple, rapid, and cost efficient, yet dependable quantitative method of analysis. In this paper, we propose a streamlined and practical approach, taking into account a critical review of several papers recently published on the subject, as well as experimental and validation data obtained in our laboratory. The resulting HPTLC method utilizes separation on silica gel 60 with cyclohexane, ethyl acetate, acetic acid (20:10:1) as mobile phase, derivatization with modified anisaldehyde reagent and highly specific densitometric evaluation of fluorescence at 520 nm with a cut-off filter at 540 nm. Over a wide concentration range of 20 ng absolute (0.05% in the dried plant material) up to a theoretical amount of 1,300 ng (3.25%), the method can be applied for screening the artemisinin content of 9 samples in an hour. Following a suitable dilution, a precise and accurate assay can be performed in the linear working range of 30–100 ng absolute.

Keywords: Artemisinin, Extraction, Assay, Densitometry, *Artemisia annua*, Validation

INTRODUCTION

Artemisinin, a natural product with antimalarial activity, is not only of great therapeutic value as a drug, but it is also of growing economic importance. Since it is difficult to synthesize, it is primarily obtained by extraction on a

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large scale from dried leaves of *Artemisia annua.* For many reasons, it is important to measure the content of artemisinin in various matrices. Sometimes it may be necessary at the same time to distinguish the compound from synthetic or semi-synthetic derivatives, or metabolites, or precursors, but initially the analytical task is to determine the extractable amount of artemisinin in the source plant. Numerous papers providing solutions for one or the other analytical question related to artemisinin have been published in the last two decades. The described techniques include TLC with densitometric evaluation[1–5] as well as HPLC with UV,[6–9] ELSD,[10,11] MS,[12–15] chemiluminescence,[16,17] and electrochemical[18,19] detection, and several GC methods with MS detection[20] or other detection modes.

The absence of a chromophore in the molecule makes detection the major challenge of any assay of artemisinin. Unless mass spectrometry or ELSD is employed, a derivatization step must be included. In thin-layer chromatography (TLC), this is a rather simple task. The technique, particularly its high-performance version (HPTLC), offers also a number of other advantages.[21] Therefore, we have reviewed earlier and some of the most recent papers[1–20] with the aim of selecting for validation the most suitable TLC approach to a simple, rapid, cost efficient, but still reliable assay of artemisinin in dried leaves.

Most of the publications focus on the separation of the target compound from various related molecules. Our approach was geared towards a baseline separation of artemisinin from all other matrix compounds that are extracted during sample preparation and a specific detection for accurate results. Another important goal of the work was to optimize all steps of the analytical process with respect to practicality and convenience ensuring broad applicability and independence from sophisticated equipment and complex laboratory settings.

EXPERIMENTAL

Material

Artemisia annua samples and artemisinin standard were supplied by X. Simonnet (Mediplant, Switzerland). Additional samples were provided by Ch. Giblain (Bionexx, Madagascar). Chemicals were purchased from Roth (Karlsruhe, Germany), and Merck (Darmstadt, Germany).

HPTLC plates silica gel 60 F_{254} were manufactured by Merck. Solvents of p.a. grade were purchased from ACROS (Gent, Belgium), Fluka (Buchs, Switzerland) or Merck. Chromatographic equipment (twin trough chamber 20×10 cm, immersion device, Automatic TLC Sampler 4, digital documentation system, TLC Scanner 3, winCATS 1.4.2 software, VideoScan 1.02 software) were made by CAMAG (Muttenz, Switzerland). Other equipment included a mill (IKA, Staufen, Germany), an ultrasonic bath (Telsonic,

Bronschhofen, Switzerland), a centrifuge (Hettich, Tuttlingen, Germany) and diverse glassware.

Preparation of Samples, Reference Solutions, and Derivatizing Reagent

Samples of dried *Artemisia annua* were finely milled. 200 mg of plant material were mixed with 10 mL of toluene and extracted by sonication for 10 min at room temperature (23°C). Following centrifugation, the supernatant was used as test solution for screening. For assay in the linear working range, the sample was diluted as necessary.

Two standard solutions were prepared. For screening, 10.1 mg of artemisinin was dissolved in 100 mL of toluene (standard solution I of 101.0 ng/μL). This solution was diluted with toluene 1:10 (standard solution II of 10.1 ng/μL) to cover the linear working range. All solutions were stored at 6°C.

Anisaldehyde reagent was prepared by adding 20 mL of acetic acid, 4 mL of sulfuric acid, and 2 mL of anisaldehyde to a mixture of 100 mL of ethanol with 80 mL of water.

Chromatography and Evaluation

The general SOP for HPTLC, as previously published,[22] was followed. Sample volumes of 2–10 μL were applied as 8 mm bands using the spray-on technique. HPTLC plates were developed with cyclohexane, ethyl acetate, acetic acid (20:10:1) over a distance of 70 mm from the lower edge of plate using a twin trough chamber, saturated for 20 min with 10 mL of mobile phase per trough. The developed plates were then dried with a hair dryer (cold air) for 5 min.

For derivatization, the plate was immersed in the reagent for 1 s. After waiting for 1 min to allow complete absorption of the reagent, the plate was heated at 100°C for 12 min. A digital image for optional video densitometry was captured under UV 366 nm.

The quantitation of artemisinin was performed by densitometric evaluation in fluorescence mode at 520 nm with cut-off filter at 540 nm using a tungsten lamp. The size of the scanning slit was adjusted to 4.00 × 0.20 mm and the scanning speed to 20 mm/s at a data resolution of 100 μm/step.

RESULTS AND DISCUSSION

The starting point for our work was the paper published by Gaudin,[2] the central element of which is a comparison and correlation of TLC and HPLC data. A thorough evaluation of the TLC method revealed two major

shortcomings. The first is associated with the derivatizing reagent (2 mL of anisaldehyde in a mixture of 200 mL of acetic acid and 4 mL of sulfuric acid). From a technical point, this variation of the classical anisaldehyde spray reagent[23] is acceptable and gives reproducible results. However, the unusually high concentration of acetic acid poses a serious problem during handling and evaluation of the derivatized plate, due to obnoxious fumes. In an attempt to find a suitable substitute, we discovered the second and more serious problem. The separation of artemisinin from other components of the sample is insufficient with the selected mobile phase hexane, diethyl ether (6:5).

When derivatized with sulfuric acid reagent (10% in methanol) and scanned in absorption mode at 530 nm, the signal intensity is generally much lower than that after derivatization with anisaldehyde reagent but, for a sample which should produce a signal within the calibration range, we found an artemisinin signal about twice as high as that obtained for the highest calibration standard (see Figs. 1a and 1b). Viewing the derivatized plate under UV 366 nm revealed, in all plant samples, the presence of a red zone co-eluting with a blue-white fluorescent zone which corresponds in color and position to that of the artemisinin reference. This red zone can also be detected in the chromatogram obtained by scanning densitometry in fluorescence mode at UV 366 nm using a 540 nm cut-off filter, which only red fluorescent light can pass (see Fig. 1c). When the reference is scanned in the same manner, no signal is detected (Fig. 1d). If densitometry is performed at UV 366 nm with a 400 nm cut-off filter, blue fluorescent light also passes. Under these conditions, a signal for artemisinin is recorded in the sample and the reference (Fig. 1e and f). The signal from the sample is now just slightly higher than that from the reference.

The mobile phase cyclohexane-ethyl acetate-acetic acid 20:10:1 proposed by Wang[1] for the determination of artemether (a derivative of artemisinin) in tablets affords sufficient separation as seen in Fig. 2a–d. With the 540 nm cut-off filter in place, neither for the sample nor for the reference is a signal detected. This means no interfering substance is present in the sample. With the 400 nm cut-off filter, the respective signals for artemisinin can be measured. Unfortunately, the intensity of the signal obtained from artemisinin by derivatization with sulfuric acid is very low. This brought us back to anisaldehyde reagent.

For a more practicable derivatization procedure, anisaldehyde reagent in ethanol, water (10:8) was selected. We followed the recommendation of Gabriëls,[3] who had evaluated various modifications and other reagents, including p-dimethylaminobenzaldehyde proposed by Wang.[1] The new derivatization results in a red zone for artemisinin which, in all previously published methods, was densitometrically evaluated by absorption measurement. The experiments described above suggested further investigation of possible fluorescence detection. Unlike sulfuric acid, anisaldehyde reagent induces a red fluorescence for artemisinin under UV 366 nm. From a

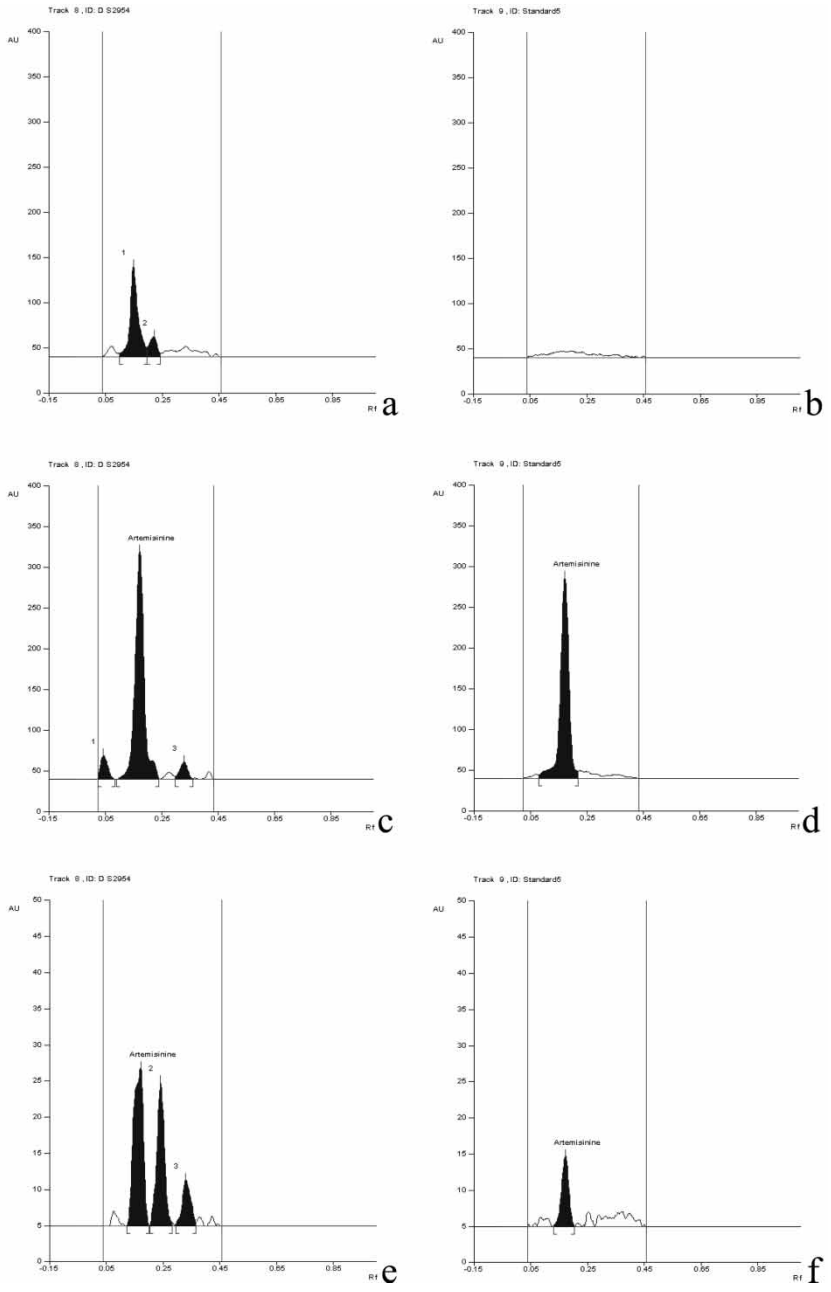

Figure 1. Detection of artemisinin in a sample (a, c, e) and as reference (b, d, f) after derivatization with 10% sulfuric acid in methanol; absorption measurement at 530 nm (a, b); fluorescence measurement at 366/>540 nm (c, d); fluorescence measurement at 366/>400 nm (e, f); for details, see text.

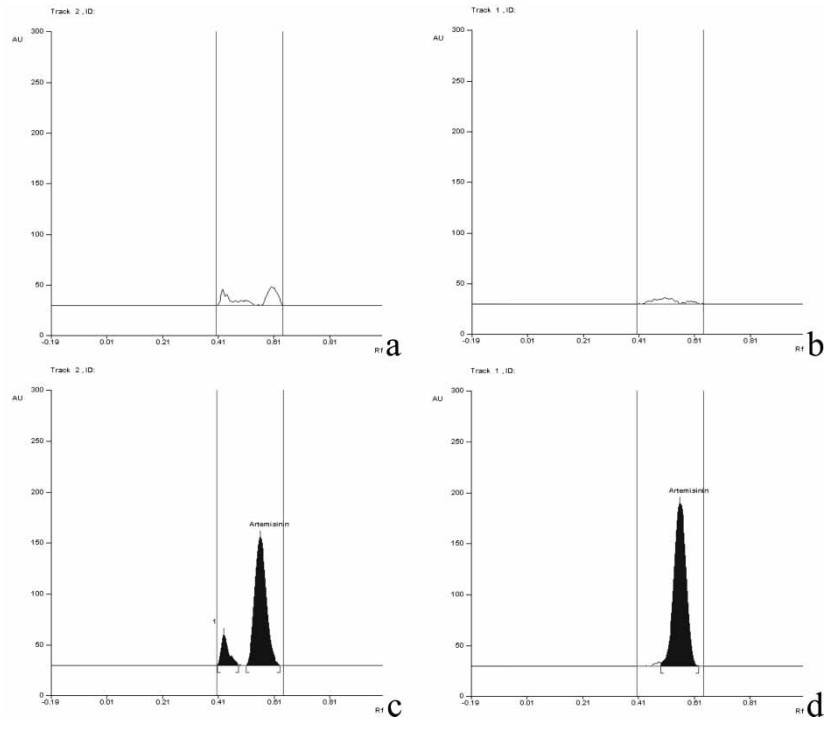

Figure 2. Detection of artemisinin in a sample (a, c) and as reference (b, d) after chromatography with modified mobile phase and derivatization with 10% sulfuric acid in methanol; fluorescence measurement at 366/>540 nm (a, b); fluorescence measurement at 366/>400 nm (c, d); for details, see text.

fluorescence excitation spectrum on the HPTLC plate, the optimum excitation wavelength of 520 nm was selected which, in combination with the 540 nm cut-off filter, enables an extremely specific detection of the target compound. Compared to a conventional absorption measurement at 535 nm, the signal obtained from the same zone in fluorescence mode is about 3 times higher.

With the new mobile phase and optimized detection procedure, stability of the analyte in the chromatographic system was established by spotting 2 μL of one sample in the corner of a 10 × 10 cm HPTLC plate. The plate was developed in two dimensions using two portions of the same mobile phase. All sample components were located on the line connecting the application position with the intersection of the fronts.

Using a Michaelis–Menten regression, it is possible to determine artemisinin in a wide concentration range from 20 ng absolute, representing 0.05% content in dried plant material, up to a theoretical amount of 1,300 ng, representing 3.25% (see Fig. 3a). A pseudo-linear working range from 30 to 120 ng absolute was established and validated (Fig. 3b). For screening samples of

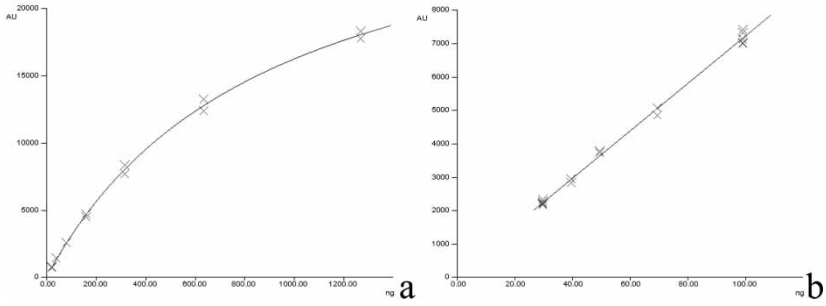

Figure 3. Calibration curve for artemisinin: a) Screening, b) linear working range.

dried plant material with unknown artemisinin content, the wide calibration range can be employed. For a second, more precise measurement, the sample can be diluted as needed to fit the pseudo-linear range. This range was the basis for further validation work with regards to precision and accuracy (see Table 1).

Precision of the analysis was investigated in several ways. For precision across the plate, 5 replicates of the same (prepared) sample were analyzed on the same plate. The relative standard deviation of the artemisinin content was calculated. For the determination of repeatability, the same (prepared) sample was analyzed in triplicate on three plates on the same day. The relative standard deviation of the mean values from each plate were calculated. Intermediate precision was investigated by comparing results for the same (prepared) sample on three plates, each being developed on a different day.

Table 1. Summary of validation data

Parameter	Value
Screening range	20–1300 ng (equal to 0.05-3.25% artemisinin) Michaelis Menten 1 fit: $y = 30722.958x/(853.168 + x)$ RSD = 3.67%
Linearity	30–120 ng (equal to 0.075–0.3% artemisinin) Linear fit: $y = 122.6 + 71.04x$ $r^a = 0.99831$; RSD = 2.92%
Precision of the analysis	5 Replicates across one plate; RSD = 0.77%
Repeatability	One aliquot in triplicate on three plates on the same day; RSD = 1.9%
Intermediate precision	One sample on three plates on three days; RSD = 1.2%
Precision of the extraction	5 Aliquots of one sample on one plate; RSD = 5.2%

r^a = correlation coefficient.

Table 2. Results of recovery study

Artemisinin in sample (ng absolute)	Spiked amount (ng absolute)	Theoretical value (ng)	Experimental value (ng)	Recovery (%)	Avg. recoveries (%)	RSD (%, n = 3)
15.39	15.45	30.8	33.17	107.6	104.2	4.81
			30.32	98.3		
			32.78	106.3		
	51.50	66.9	70.43	105.3	103.8	6.75
			64.37	96.2		
			73.6	110.0		
	82.40	97.8	90.74	92.8	100.2	8.88
			95.54	97.7		
			107.63	110.1		

Between analyses, the remainder of the sample was stored in the refrigerator at 6°C. Inter-laboratory precision was not investigated.

The accuracy of the quantitation was assessed in a recovery study (see Table 2). A sample with known artemisinin content was diluted, applied in triplicate onto the plate (2 μL), and individually spiked with three different amounts of artemisinin to reach a final content in the lower, middle, and upper range of the pseudo-linear calibration. Accuracy was further investigated by analyzing the same (prepared) sample using other stationary and mobile phases, as well as other detection modes. Results are compared in Table 3. With one exception, good agreement of the measurements was found. The value obtained with system 4 is too high and suggests a systematic

Table 3. Assessment of accuracy using different stationary and mobile phases

System		Detection mode	Artemisinin content (%)
1	New method	Fluorescence 520/>540 nm	0.666
2	New method	Absorption 535 nm	0.689
3	Method according to Gabriëls,[4] layer RP18, mobile phase acetonitrile, water (2:1)	Fluorescence 520/>540 nm	0.648
4	Method according to Bhandari,[5] layer RP18, mobile phase 0.2% TFA in water, acetonitrile (7:13)	Fluorescence 520/>540 nm	0.928

Table 4. Evaluation of sample extraction

Extraction procedure	Extraction solvent	Artemisinin content (%)	Source of method
Sonication 10 min (first extraction)	Toluene	0.823	Gaudin[2]
Sonication 10 min (subsequent extraction)	Toluene	0.007	
Sonication 10 min	Pentane	0.524	
Sonication 10 min	Acetonitrile	0.672	
Soxhlet extraction	Pentane	0.428	
Immersion for 1 min	Chloroform	0.643	Van Nieuwerburgh[14]
Keep overnight in	n-Hexane	0.521	Bhandari[5]

error. The use of reversed phase (systems 3 and 4) was not further considered because plates are considerably more expensive than regular HPTLC plates coated with silica gel 60.

Finally, we looked at the precision of the entire procedure including the sample extraction. To verify a complete extraction, portions of the same sample were extracted with different solvents, several times with the same solvent, and continuously in a Soxhlet apparatus. Results can be found in Table 4. Toluene as extraction solvent and sonication for 10 min proved to be the most suitable and exhaustive method of sample preparation. For this extraction, the precision was determined by preparing 5 aliquot portions of the same sample for analysis on the same plate. Relative standard deviation was 5.2%.

CONCLUSION

HPTLC on silica gel 60 with cyclohexane, ethyl acetate, acetic acid (20:10:1) with densitometric evaluation of fluorescence at $520/>540$ nm following derivatization with anisaldehyde reagent in ethanol, water (10:8) is a simple, rapid, accurate, and precise method for determination of artemisinin extracted with toluene by sonication of powdered dried leafs of *Artemisia annua.*

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REFERENCES

1. Wang, Y.L.; Ding, J.X. Studies on the stability of artemether tablet by thin- layer chromatography (Chinese). J. Chinese Herb Med. (Zhongcaoyao) **1989**, *20*, 297–299.
2. Gaudin, M.; Simonnet, X. Dosage de l'artémisinine par chromatographie sur couche mince (CCM). Rev. Suisse Vitic. Arboric. Hortic. **2002**, *34* (3), 205–208.
3. Gabriëls, M. Plaizier-Vercammen, J. Densitometric thin-layer chromatographic determination of artemisinin and its lipophilic derivatives, artemether and arteether. J. Chromatogr. Sci. **2003**, *41* (7), 359–366.
4. Gabriëls, M.; Plaizier-Vercammen, J. Development of a reversed-phase thin-layer chromatographic method for artemisinin and its derivatives. J. Chromatogr. Sci. **2004**, *42* (7), 341–347.
5. Bhandari, P.; Gupta, A.P.; Singh, B.; Kaul, V.K. Simultaneous densitometric determination of artemisinin, artemisinic acid and arteannuin-B in *Artemisia annua* using reversed-phase thin-layer chromatography. J. Sep. Sci. **2005**, *28* (17), 2288–2292.
6. Zhao, S.S.; Zeng, M.Y. Spektrometrische Hochdruck-Flüssigkeits-Chromatographische (HPLC) Untersuchungen zur Analytik von Qinghaosu. Planta Med. **1985**, *51*, 233–237.
7. El Sohly, H.N.; Croom, E.M.; El Sohly, M.A. Analysis of the antimalarial sesquiterpene artemisinin in *Artemisia annua* by high-performance liquid chromatography (HPLC) with postcolumn derivatization and ultraviolet detection. Pharm. Res. **1987**, *4* (3), 258–260.
8. Vandenberghe, D.R.; Vergauwe, A.N.; Van Montagu, M.; Van den Eeckhout, E.G. Simultaneous determination of artemisinin and its bioprecursors in *Artemisia annua*. J. Nat. Prod. **1995**, *58* (5), 798–803.
9. Qian, G.P.; Yang, Y.W.; Ren, Q.L. Determination of artemisinin in *Artemisia annua* L. by reversed phase HPLC. J. Liq. Chromatogr. Rel. Technol. **2005**, *28* (5), 705–712.
10. Liu, C.Z.; Zhou, H.Y.; Zhao, Y. An effective method for fast determination of artemisinin in *Artemisia annua* L. by high performance liquid chromatography with evaporative light scattering detection. Anal. Chim. Acta **2007**, *581* (2), 298–302.
11. Avery, B.A.; Venkatesh, K.K.; Avery, M.A. Rapid determination of artemisinin and related analogues using high-performance liquid chromatography and an evaporative light scattering detector. J. Chromatogr. B **1999**, *730* (1), 71–80.
12. Wang, M.; Park, C.H.; Wu, Q.; Simon, J.E. Analysis of artemisinin in *Artemisia annua* L. by LC-MS with selected ion monitoring. J. Agric. Food Chem. **2005**, *53* (18), 7010–7013.
13. Bilia, A.R.; Melillo de Malgalhaes, P.; Bergonzi, M.C.; Vincieri, F.F. Simultaneous analysis of artemisinin and flavonoids of several extracts of *Artemisia annua* L. obtained from a commercial sample and a selected cultivar. Phytomedicine **2006**, *13* (7), 487–493.
14. Van Nieuwerburgh, F.C.W.; Vande Casteele, S.R.F.; Maes, L.; Goossens, A.; Inzé, D.; Van Bocxlaer, J.; Deforce, D.L.D. Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography-electrospray quadrupole time-of-flight tandem mass spectrometry. J. Chromatogr. A **2006**, *1118* (2), 180–187.
15. Naik, H.; Murry, D.J.; Kirsch, L.E.; Fleckenstein, L. Development and validation of a high-performance liquid chromatography—mass spectroscopy assay for

determination of artesunate and dihydroartemisinin in human plasma. J. Chromatogr. B **2005**, *816* (1–2), 233–242.
16. Green, M.D.; Mount, D.L.; Todda, G.D.; Capomacchia, A.C. Chemiluminescent detection of artemisinin novel endoperoxide analysis using luminol without hydrogen peroxide. J. Chromatogr. A **1995**, *695* (2), 237–242.
17. Amponsaa-Karikari, A.; Kishikawa, N.; Ohba, Y.; Nakashima, K.; Kuroda, N. Determination of artemisinin in human serum by high-performance liquid chromatography with on-line UV irradiation and peroxyoxalate chemiluminescence detection. Biomed. Chromatogr. **2006**, *20* (11), 1157–1162.
18. Sandrenan, N.; Sioufi, A.; Godbillon, J.; Netter, C.; Donker, M.; Van Valkenburg, C. Determination of artemether and its metabolite, dihydroartemisinin, in plasma by high-performance liquid chromatography and electrochemical detection in the reductive mode. J. Chromatogr. B **1997**, *691* (1), 145–153.
19. Na-Bangchang, K.; Congpuong, K.; Hung, L.N.; Molunto, P.; Karbwang, J. Simple high-performance liquid chromatographic method with electrochemical detection for the simultaneous determination of artesunate and dihydroartemisinin in biological fluids. J. Chromatogr. B **1998**, *708* (1–2), 201–207.
20. Mohamed, S.S.; Khalid, S.A.; Ward, S.A.; Wan, T.S.M.; Tang, H.P.O.; Zheng, M.; Haynes, R.K.; Edwards, G. Simultaneous determination of artemether and its major metabolite dihydroartemisinin in plasma by gas chromatography-mass spectrometry-selected ion monitoring. J. Chromatogr. B **1999**, *731* (2), 251–260.
21. Reich, E.; Schibli, A. *High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants*; Thieme Medical Publishers, Inc.: New York, USA, 2007, 13.
22. Reich, E.; Schibli, A. A standardized approach to modern high performance thin-layer chromatography (HPTLC). J. Planar Chromatogr **2004**, *17* (6), 438–443.
23. Jork, H.; Funk, W.; Fischer, W.; Wimmer, H. *Thin-Layer Chromatography;Reagents and Detection Methods*; VCH Verlagsgesellschaft mbH: Weinheim, Germany, 1990; Vol. 1a, 196pp.

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