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Development of HPLC analytical protocols for quantification of artemisinin in biomass and extracts

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ABSTRACT

Quantification of artemisinin purity and amount in plant material and extracts to date has been characterized by a considerable inconsistency in values. This is likely to be due to the adoption of varied analytical procedures and use of inappropriate to the specific applications analytical techniques. In this paper we are attempting to further develop artemisinin analysis to the point where a universally acceptable reference method is available to the research and end-users communities. Thus, we have developed and validated an HPLC-RI method and optimized an HPLC-ELSD method. We used the gradient HPLC-UV method recommended by the current artemisinin monograph as a comparison for the method improvements presented herein, and show the limitations for its application scope. The data reported should help to allow more reliable laboratory analysis of artemisinin in both pure samples and in *Artemisia annua* extracts.

1. Introduction

Artemisinin (**1**) derived antimalarial drugs have been in widespread use for over a decade and are still produced solely from the artemisinin obtained by extraction from *Artemisia annua L.* [1]. Their use as combination therapies (artemisinin combination therapy, ACT) is rapidly expanding, following advice from the World Health Organisation (WHO). Consequently, the artemisinin supply chain is becoming more robust with more farmers, extraction companies and ACT manufacturers entering the field. The areas of cultivation of *A. annua* and the number of processing sites are also expanding beyond the traditional producing regions of China and Vietnam into Brazil, Argentina, India, Europe, and notably into African countries, where the problem of malaria is most prominent. However, many small companies and new-comers into the field of growing *A. annua* and the extraction of artemisinin have limited in-house R&D and analytical expertise. With the expanding number of players within the field of naturally derived artemisinin, there is an apparent and developing inconsistency in the bulk artemisinin quality, which is exacerbated by the apparent inconsistencies in the analytical procedures used. This problem has been highlighted at a number of recent conferences dedicated to artemisinin [2–4].

A number of analytical procedures to quantify the amount of artemisinin in the collected plant material, in the primary and purified extracts, and to quantify its purity as a bulk drug precursor have already been reviewed [5,6]. Since these reviews a GC-MS method, and the principle component methodology for the analysis of the development of a metabolic profile of *A. annua* during different stages of growth were reported [7]. A proton NMR method of quantification was also proposed [8], although no validation data (limits of detection/quantification and precision) were reported. A modification to the LC-MS method was reported, establishing a very high sensitivity to artemisinin [9].

The WHO's monograph on *A. annua* cultivation [10] and the artemisinin monograph by the International Pharmacopeia [11] contain descriptions of several analytical methods. The simplest method is based on thin layer chromatography (TLC). Although TLC is most frequently used as a qualitative tool, quantitative and preparative TLC methods are available [12]. It has now been widely accepted in the field of artemisinin, that TLC method consistently

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Table 1
The conditions for HPLC analysis based on the International Pharmacopeia artemisinin monograph [11].

Time (min)	Acetonitrile (%, v/v)	Water (%, v/v)	Comment
0–17	60	40	Isocratic
17–30	60 → 100	40 → 0	Linear gradient
30–35	100 → 60	0 → 40	Return to initial conditions
35–45	60	40	Isocratic re-equilibration

The column is 100 mm × 4.6 mm with a 3 μm particle stationary phase (*the nature of the stationary phase is not specified in the Monograph*). The recommended flow-rate of the mobile phase is 0.6 mL min^{-1} and detection is by UV at 216 nm.

underestimates the amount of artemisinin when compared with HPLC methods.

The most common method for analysis of artemisinin is based on high performance liquid chromatography (HPLC). The monographs [10,11] describe an HPLC method using UV detection at 214 nm and dihydroartemisinin (**5**) as an internal standard, see Table 1. The 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.

Many authors discard the WHO HPLC method on the basis of very low UV absorbance of artemisinin [5,6,13–16]. It was hence suggested to use pre- or post-column hydrolysis of artemisinin into more UV-active compounds to allow the use of the most widespread HPLC-UV instruments [14,16,17]. There are also reports on using other detectors, such as electrochemical reduction [18,19] and evaporative light scattering (ELSD) [15,20]. However, the issues surrounding accurate determination of artemisinin quantity in different situations cannot be resolved solely by replacing the detector on an LC system.

Apart from the analysis of pure bulk artemisinin that can be done using WHO monograph HPLC method, quantification of artemisinin in the leaf and at different stages of the production of bulk artemisinin as a drug precursor is also required. This process is complicated by the large number of co-extracted compounds and very close structural co-metabolites in the plant. An earlier method describes co-determination of the key compounds of *A. annua* (**1–4**) by using HPLC with UV and electrochemical detection methods and using dry column chromatography to purify the extracts [18]. However, details of the extract preparation are still sketchy and comparison of extraction solvents was limited only to aliphatic solvents. It was also reported in the literature [5], that HPLC methods do not separate artemisinin from deoxyartemisinin (**5**), which results in an overestimation of the artemisinin content in pure samples.

Thus, the purpose of this study was to systematically address the issue of HPLC methodology for quantification of artemisinin in three key areas: (i) analysis of artemisinin and co-products content in plant material, (ii) analysis of artemisinin and its co-products in extracts, and (iii) analysis of artemisinin impurities in the "pure" bulk artemisinin samples Scheme 1.

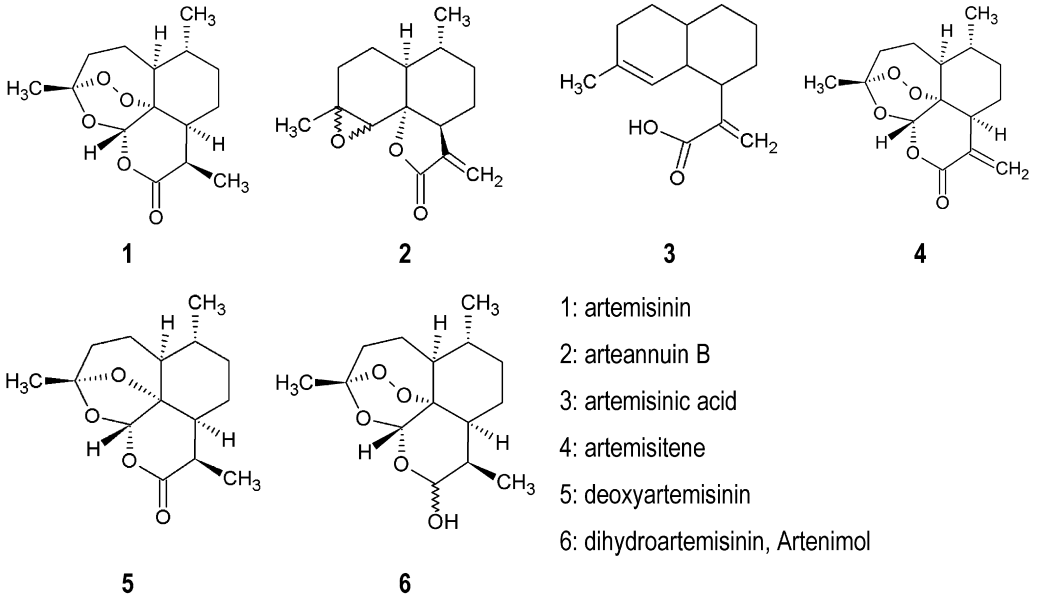
2. Experimental

2.1. Instrumentation

All solvents used in HPLC experiments were "HPLC grade" and from a number of sources. An HPLC instrument (Shimadzu Prominence) equipped with a UV–vis diode-array (SPD-M20A, DAD) and in line evaporative light scattering (ELSD, LTII, 350 kPa N_2, nebulizer at 40 °C) detectors were used for validating the earlier published protocols and for the optimization of the mobile phases and column conditions of the ELSD-based protocol. The optimal protocol is based on a 50:30:20 (%, v/v) acetonitrile:water:methanol mobile phase with a column temperature of 45 °C. Columns used were: (1) Shimadzu XR-ODS 50 mm × 2 mm with a 2.2 μm deactivated type B silica, 12 nm pores at a column flow-rate 0.5 mL min^{-1} and (2) a Betasil C18 5 μm 250 mm × 4.6 mm at column flow-rate 1.0 mL min^{-1}.

A Dionex Ultimate 3000 HPLC instrument equipped with an RI detector (Shodex RI-101) was used to develop a new protocol based on refractive index detection. A Phenomenex Gemini 5 μm C18 11 nm 250 mm × 4.6 mm column with a guard column was used. The mobile phase was 60:40 (%, v/v) acetonitrile:water at 1.0 mL min^{-1} at ambient column temperature.

A Gilson HPLC instrument equipped with a UV (Spectromonitor 3000 LDC/Milton Roy, set at 220 nm) and ELSD (PL-EMD 960 by Polymer Laboratories) detectors in line was used to screen different columns and different mobile phases. Details of these are given in Tables 2 and 3. ELSD nitrogen flow-rate was 3.6 L min^{-1} and evaporation temperature 55 °C.



Scheme 1. Chemical structures of artemisinin co-metabolites and key co-extracted impurities.

The UV–vis spectra of artemisinin and extracts were recorded using a Shimadzu UV-1601 dual beam spectrophotometer.

The LC-MS measurements were performed using an Agilent 1200 HPLC and a Bruker Daltonics microTOF ESL mass spectrometer.

2.2. Samples preparation

Several different samples of pure artemisinin were used for this study. Artemisinin samples were (a) purchased from Sigma–Aldrich, (b) provided by Neem Biotech Ltd. (Newport, Wales, UK), (c) provided by Medicines for Malaria Ventures (MMV), and (d) provided by Hung Thinh Co (Vietnam). The plant material used was sourced from East Africa (East African Botanicals), Tasmania (GlaxoSmithKline Australia) and Argentina (Mundo Sano Foundation).

Several different solvent extracts were used for this study. For development of the HPLC-RI method chloroform extracts were used. Chloroform *A. annua* extracts were prepared from 3 g dried plant tissue extracted with 80 mL chloroform for 10 min with stirring at room temperature. The extract was stripped of solvent *in vacuo* and re-dissolved in 5 mL mobile phase. An equal volume of internal standard (β-artemether prepared in mobile phase at 2.5 mg mL^{-1}) was added to the extract. Samples were filtered through a 0.2 μm syringe filter before injecting onto the column. A larger than standard 100 μL injection loop was used.

For HPLC-ELSD and -UV studies chloroform, hexane, acetonitrile and ethyl acetate extracts were prepared using the ratio of 10 g of *A. annua* dry leaf ground to fine particles to 100 mL of solvent. After extraction solvent was evaporated *in vacuo* and residue re-dissolved in 20 mL acetonitrile. The extracts were filtered through 2 μm nylon membranes prior to injection into HPLC.

3. Results and discussion

3.1. Optimization of stationary phase; column selection

In the process of selecting the best stationary phase and column length for analysis of *A. annua* extracts and of pure bulk artemisinin, standard TMS capped C18 columns were compared with columns with shorter chain groups, polar and aromatic groups. For the C18 columns (see Tables 2 and 3), columns # 1, 6, 12–15, the order of elution of the three reference compounds in increasing retention times was artemisitene < deoxyartemisinin < artemisinin. Artemisitene was separated by almost 2 min from deoxyartemisinin and artemisinin. However, separation of the latter two compounds was much smaller with baseline separation requiring an elution time difference of at least 0.4 min. The observed differences in separation were primarily due to differences in the C18 stationary phases between column manufacturers.

Columns with capping groups other than C18 (entry 2–6, see Table 2) were also investigated. Separation of the reference compounds decreased with decreasing chain length of the hydrocarbon bonded to the stationary phase. Thus, C1(8) and CN(10) were much

Table 2
HPLC-ELSD analysis of artemisinin, artemisitene and deoxyartemisinin using 150 mm × 4.6 mm columns.

ID	Column type	Retention times (min)			Retention times (min)			Retention times (min)		
		75% Aqueous methanol			75% Aqueous acetonitrile			50% Aqueous acetonitrile		
		(4)	(1)	(5)	(4)	(1)	(5)	(4)	(1)	(5)
1	Luna 5 μm C18(2)	4.2	5.8	6.1	3.8	4.5	4.7	12.2	15.8	
2	Synergi 4 μm Polar RP	6.5	7.8	7.0	3.4	3.7		10.3	11.2	10.3
3	Synergi 4 μm Max-RP 80A	4.0	5.4	5.7	2.9/3.8			11.4	14.6	
4	Synergi 4 μm Hydro-RP 80A	4.6	6.5	6.8	3.9	4.6	4.9	13.3	17.0	
5	Synergi 4 μm Fusion-RP 80	3.4	6.4		4.0	4.5/4.7		11.5	14.2	
6	Kingsorb 5 μm C18	4.7	6.7	7.0	4.9	5.9	6.2	12.0	15.7	
7	Luna 5 μm Phenyl–hexyl	6.8	8.9	8.2	3.6	3.9		10.8	11.8	10.8

Table 3
HPLC-ELSD analysis of artemisinin, artemisitene and deoxyartemisinin using 250 mm × 4.6 mm columns.

ID	Column type	Retention times (min)			Retention times (min)			Retention times (min)			Retention times (min)		
		75% Aqueous methanol			85% Aqueous methanol			75% Aqueous acetonitrile			85% Aqueous acetonitrile		
		(4)	(1)	(5)	(4)	(1)	(5)	(4)	(1)	(5)	(4)	(1)	(5)
8	S5C1		4.3						6.3				
9	S5C8	5.7	6.8	7.1				4.7	5.1/5.4				
10	EXCN		4.4						5.7*				
11	LispOH		3.8†					3.0	3.2/3.3				
12	Luna 5 μ C18(2)	7.4	10.1	10.7	5.0	6.5	7.4	8.1	9.7	10.1	5.4	6.2	6.6
13	INODS 2	6.6	9.1	9.5				4.3	6.7	7.1			

* No separation with 50% aqueous acetonitrile therefore 70% aqueous acetonitrile not tested.

† No separation even with 60% methanol solvent.

Table 4
Mobile phases for an isocratic HPLC-ELSD method.

	Mobile phase	pH	CV* of retention time (%)	
			Standard	Extract
A	Acetonitrile/aqueous ammonium hydroxide (40:60%, v/v), pH adjusted with acetic acid	5.5	0.20	9.0
B	Acetonitrile/water/methanol (50:30:20%, v/v)	8.4	0.06	0.25
C	Acetonitrile/water/methanol (40:45:15%, v/v) and 0.1% acetic acid	4.9	0.76	0.47
D	Acetonitrile/10 mM ammonium acetate (65:35%, v/v) and 0.1% acetic acid	5.9	3.0	6.0
E	Acetonitrile/water (35:65%, v/v); water adjusted to pH 3 by adding trifluoroacetic acid (TFA)	3	0.90	0.30
F	Acetonitrile/water (60:40%, v/v) pH not adjusted	8	0.20	0.20

* CV ≡ 'coefficient of variation', or relative standard deviation expressed in percent.

worse than C8(9), which in turn was worse than C18. End-capping with polar groups (4 and 5) or a TMS group (3 and 6) did not significantly alter the selectivity of these C18 columns. Columns with aromatic groups, such as phenyl–hexyl (7) and ether-linked phenyl groups (2), significantly reduced the retention time of deoxyartemisinin so that it eluted before artemisinin (opposite to that observed for all other columns tested here).

Mobile phase composition and column length also influenced the separation observed on the studied columns. Almost baseline separation of all three reference compounds was achievable on C18 250 mm (columns 12 and 13) and 150 mm (columns 1, 6 and 7) with all three combinations of methanol, acetonitrile and water, the solvent composition being more critical for the shorter columns. Thus, good separation was observed between deoxyartemisinin and artemisinin with 75% aqueous acetonitrile or methanol, but was negligible when eluted with 50% aqueous acetonitrile. A number of combinations of acetonitrile, water and methanol were investigated and a ratio of 5:3:2 considered to be the best compromise for separation of compounds, peak shapes and in detection/quantification of other metabolites when present in low concentrations in the extracts.

Increasing column temperature decreased retention times and sharpened the peaks. However there appeared to be little improvement in separation of the three reference compounds. For quantitative determinations (to achieve good reproducibility and improve signal to noise ratio due to peak sharpening) we used constant column temperature of 40 °C, whenever possible.

Concluding the screening of different columns in combinations with mobile phases, best separation, especially for the case of deoxyartemisinin and artemisinin was achieved on columns with aromatic groups bonded to the stationary phase (2 and 7). However they are inferior to standard C18 columns for profiling plant extracts. C18 columns differed in performance. The 250 mm columns were superior in performance to the 150 mm columns for the same stationary phase. Of the four 250 mm columns tested under these conditions, Synergy Luna (12) and Betasil (15) gave better separations of the three standards and profiling of the metabolites in plant extract than Kingsorb (14) and INDOS2 (13). Aqueous methanol generally gave better separation of the reference compounds than aqueous acetonitrile. However, artemisinin is significantly more soluble in acetonitrile than in methanol. A combination of acetonitrile, water and methanol (50:30:20%, v/v) was found to be the most effective for the HPLC analysis of *A. annua* extracts, giving better peak shapes and resolution of artemisinin from impurities. This mixture also allows a wider range of compounds to be dissolved in the injection solvent so that profiling of extracts is more representative.

3.2. Optimization of the mobile phase

Mobile phase optimization was done also in terms of stability of retention time, as well as achieving correct order of elution and good baseline separation. Apart from the isocratic stage of the protocol described in Table 1, we have investigated five other options of mobile phases, all listed in Table 4. All experiments were performed using the ELSD detection.

These experiments were performed using a short (50 mm) column. The best reproducibility of residence time for the standard sample of artemisinin (Neem Biotech Ltd. sample) and for the hexane extract was found with the acetonitrile:water:methanol mobile phase (B), with the coefficient of variation (CV) of 0.06% and 0.25% for the standard and the extract respectively. The worst results were obtained with mobile phases A and D, resulting in CV values for extracts of 9% and 6% respectively.

Variation of pH and mobile phase results in a significant change in the absolute retention time of artemisinin, which than also influences the separation of by-products. Fig. 1 shows the results of analysis of artemisinin standards using mobile phases listed in Table 4.

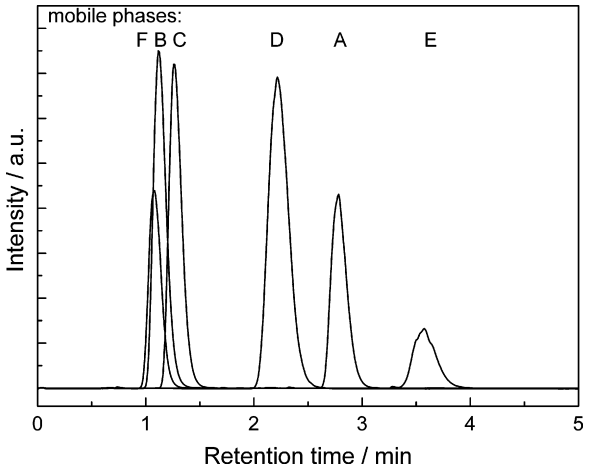


Fig. 1. The influence of mobile phase on the retention time of artemisinin standard. Shimadzu XR-ODS 50 mm × 2 mm column, 0.5 mL min^{-1} flow-rate, column temperature 45 °C and ELSD detector were used. The legend corresponds to Table 4.

Mobile phases B, C and F, containing acetonitrile with concentrations between 35% (v/v) and 60% (v/v) and pH varying between 4.9 and 8.4, all have very similar short retention times between 1.1 and 1.3 min. Between the two mobile phases containing methanol (B and C) pH does not appear to have a significant influence on the retention time, with the more acidic mobile phase resulting in only slightly longer residence time. The mobile phase without methanol and without adjustment of pH gives a similar residence time, but the presence of methanol improves the peak shape, resulting in a sharper elution of artemisinin.

The most significant influence of pH was observed between the two mobile phases containing acetonitrile and water only, E and F. The very acidic mobile phase results in a poor peak shape and a significant increase in retention time.

The two mobile phases containing ammonium hydroxide and ammonium acetate with similar pH give intermediate values of retention time, with the same order of the effect of pH—the more acidic mobile phase results in a longer retention time.

The short 50 mm column was only used for qualitative development of mobile phases, since it did not allow good separation of closely related components of extracts and especially of the peaks near the artemisinin peak; hence all following work was performed using the longer columns.

3.3. Suitability of UV detection for artemisinin quantification

The HPLC method described in the current artemisinin monograph [11] is based on UV detection without any derivatisation of artemisinin. However, a number of literature sources mention that artemisinin is a weak chromophor and therefore its UV analysis is impossible. Yet, within pharmaceutical companies there are also validated protocols for HPLC-UV analysis of the artemisinin-based APIs that include artemisinin as an impurity [21].

Based on the concentration dependent UV absorption data between 205 and 215 nm, we estimated the molar absorption coefficient of artemisinin to be between 163 and 183 L mol^{-1} cm^{-1}, with the maximum at 210 nm. The value of extinction coefficient of artemisinin is indeed very low in comparison to strongly absorbing molecules. Thus, the upper range of extinction coefficient 10^5 L mol^{-1} cm^{-1} is known for organic dyes. A comparison of extinction coefficients of artemisinin with those

of its co-metabolites and co-extracted compounds is given in [22].

The range of UV absorption between 205 and 215 nm is not very specific, since many compounds, including solvents, would absorb in this range. This also applies to *A. annua* extracts, where many compounds in the extract would absorb stronger than artemisinin at these wavelengths. Therefore, if chromatographic separation is imperfect, the UV detection would not allow good quantification of artemisinin due to the difficulty in identifying the small peak of artemisinin among others. Therefore, UV detection at the end of HPLC instrument is most suitable for quantitative analysis of bulk purified artemisinin (within the constraints of the limits of detection of impurities), and not for the analyses of extracts and leaf composition profile.

The spectrum of a plant extract also shows absorption maxima at 273, 327, 410, 534, 607 and 664 nm. Standards of compounds **1**, **3**–**6** were analyzed using a diode-array UV detector and none exhibited absorbance at these wavelengths. Hence, the absorbance maxima observed in the extract correspond to other than the artemisinin-class compounds, and multivariant analysis based on the diode-array UV detection would not be useful. However, it is worth mentioning that a diode-array UV detection at 210 and 280 nm was recently employed for simultaneous determination of artemisinin and a flavone in *A. annua* extracts [23].

3.4. Comparison of UV and ELSD quantification of pure bulk artemisinin

A direct comparison between UV and ELSD methods of detection was made using acetonitrile dissolved artemisinin standard solution. In these experiments we used two mobile phases, containing (i) acetonitrile:water:methanol (50:30:20%, v/v), and (ii) acetonitrile:water (65:35%, v/v).

The UV detection calibration was found to be linear over a wide range of concentrations, at least within 0.1–10 mg mL^{-1}, whereas the ELSD detector is inherently non-linear, best fitted either by several linear relationships over narrow ranges of concentration, or by a polynomial or a power relationship over a broad range of concentrations. The linearity determined over 0.1–10 mg mL^{-1} range is better in the case of UV detection, see Table 5.

The UV detector used also exhibited a better signal to noise characteristic, which allowed a lower limit of quantification in comparison with the ELSD detector. The relevant International Conference on Harmonization guidelines were used for structuring the validation experiments [24]. The limit of quantification was set to be the peak height 10 times that of noise, whereas the limit of detection was set as 3 times that of noise peak height. The obtained value of the quantification limit 0.1 mg mL^{-1} is consistent with that found earlier for the ELSD detection method [15]. This value is, however, much worse than the limit of detection for an HPLC-ELSD method reported earlier [20] at *ca*. 0.003 mg mL^{-1} (the corresponding limit of quantification should be around 0.01 mg mL^{-1}, i.e., one order of magnitude better than found in this study, and in [15]). This is most likely due to the very strong sensitivity of the ELSD to the set-up and preparation. The warm-up time, stabilization time, nebulizer temperature, stability of carrier gas pressure and flow all have very significant effect on the ELSD performance.

The ELSD detection appears to show better characteristics when used in conjunction with the isocratic LC mode. This was further investigated in terms of repeatability, and inter day and intra day intermediate precision. Repeatability was calculated based on six consecutive injections of artemisinin standard with concentration 10 mg mL^{-1}. Intra day intermediate precision was calculated on the basis of six injections made at different times on the same day. Inter day precision was calculated on the basis of six injections made on two consecutive days at different times and by two different analysts.

The isocratic methods using the ELSD detector appear to have better repeatability of retention time, which is an important factor in the case of identification and quantification of artemisinin in the extracts (see Table 6). Between the two isocratic methods using different mobile phases, the mobile phase containing methanol gives a marginally worse inter day precision and a wider concentration accuracy window. The UV-based gradient method gives the highest concentration precision and good accuracy for the concentrated standard solutions in comparison with the ELSD-based isocratic methods.

The accuracy of the HPLC-UV and HPLC-ELSD methods does not compare well with the required purity of artemisinin by the current monograph, requiring that artemisinin content by HPLC-UV is within 97–102% [11]. The gradient HPLC-UV method and the isocratic HPLC-ELSD method with acetonitrile:water (65:35%, v/v) mobile phase provide just enough accuracy for the monograph standard. However, in order to detect any impurities with a lower content, i.e., within the last ±2%, a different, more sensitive method is required, for example, an LC-MS.

3.5. Development of HPLC-RI method

Refractive index detection is routinely used in HPLC methods for analysis of sugars and alcohols, or more generally of low UV absorbing species. Its use for artemisinin analysis has not been disclosed or validated in the accessible prior literature. All experiments were performed using Gemini C18 250 mm × 4.6 mm column and acetonitrile:water (60:40%, v/v) mobile phase. By varying the ratio of acetonitrile:water between 55% and 75% (v/v) of acetonitrile in the isocratic experiments, it was confirmed that 60% (v/v) acetonitrile gives the best resolution of peaks in the analysis of extracts.

Linearity of analysis was checked with both the reference standard and the chloroform extracts, confirming that the linearity range is between 0.025 and 20 mg mL^{-1}. The lower limit of 0.025 mg mL^{-1} corresponds to the detection limit, set as 2.5:1 signal to noise ratio. The quantification limit was set as 0.1 mg mL^{-1}, corresponding to 10:1 signal to noise ratio. Thus, the working quantification range for the HPLC-RI method is 0.1–20 mg mL^{-1}

Table 5
Comparison of linearity and limits of detection and quantification of UV and ELSD detection of artemisinin.

Method	Linearity over 0.1–10 mg mL^{-1}		Limit of detection		Limit of quantification	
	R	R^2	mg mL^{-1}	CV (%)	mg mL^{-1}	CV (%)
Gradient UV[a], 213 nm	0.9999	0.9999	0.009	6.7	0.020	10.0
Gradient ELSD[a]	0.9970	0.9950	0.001	9.2	0.100	3.1
Isocratic UV[b], 210 nm	0.9999	0.9999	0.001	0.25	0.009	0.7
Isocratic ELSD[b]	0.9990	0.9979	0.020	0.7	0.100	2.4

[a] Mobile phase: see Table 1.
[b] Mobile phase: 50:30:20 acetonitrile:water:methanol, % (v/v).

Table 6
Comparison of intermediate precision of HPLC-UV (gradient), and HPLC-ELSD (isocratic) methods.

Mobile phase/method	Retention time CV (%)		Repeatability of retention time CV (%)	Concentration CV (%)		Concentration accuracy (%)	
	Intra day	Inter day		Intra day	Inter day	Intra day	Inter day
Gradient, see Table 1 for conditions	0.54	0.87	0.47	0.16	0.27	99.4	98.8
Isocratic, 50:30:20% (v/v) acetonitrile:water:methanol	0.06	0.40	0.04	0.25	7.6	96.0	110.0
Isocratic 65:35% (v/v) acetonitrile:water	0.03	0.10	0.04	0.97	6.34	100.8	105.6

Table 7
Summary of characteristics of the HPLC-RI method of artemisinin detection and quantification.

Linearity (R^2)	Repeatability, CV (%)	Limit of detection (mg mL^{-1})	Limit of quantification (mg mL^{-1})
0.9995	6	0.025	0.1

concentration of artemisinin (see Table 7). This is identical to the HPLC-ELSD method reported in the literature [15], and as confirmed in this study, see Table 5.

Both, repeatability and precision were analyzed in terms of the coefficient of variation. Repeatability of measurements within a day-long assay was found to be better than 6%, where the coefficient of variation was calculated from the means of three data sets each consisting of three assays. The intermediate precision, which is defined as a coefficient of variation of a series of assays performed in the same laboratory over different days and by different analysts, was found between 0.02% and 41%. An apparent exponential correlation of the loss of precision with the decrease in the mean concentration of artemisinin in the samples was found. Thus, for the worst rate of degradation of an artemisinin sample (a sample in the analysis solvent prepared for HPLC assay and stored for a period of time at a given temperature; observed degradation rate was 1.7×10^{-2} h^{-1}) at initial artemisinin concentration of 0.2 mg mL^{-1} would result in the decrease in concentration to 0.102 mg mL^{-1} over a 48 h period, thus giving an error of at least 50% coming only from the degradation of artemisinin. This explains the very significant error for the inter day precision at low concentrations of artemisinin. It is worth noting that poor stability of artemisinin in solutions is a known problem, see, e.g. [25], and procedure for keeping standards at 4 °C not longer than three days was reported in [20], but not investigated systematically.

3.6. Analysis of *A. annua* extracts

It has been widely speculated that some impurities of artemisinin present in extracts, and in crystals, are not readily separated by HPLC methods. This causes problems not only at the stage of developing an extraction method, but also in terms of quality control of the produced bulk artemisinin. The ELSD traces of *A. annua* extracts obtained using two different mobile phases, and the corresponding ELSD traces of standards are shown in Fig. 2. A comparison of UV and ELSD signals of ethyl acetate extracts of *A. annua* is shown in Fig. 3. The analysis was performed using a Shimadzu Prominence instrument. We also used LC-MS analysis to confirm the correspondence of peaks to compounds in the complex extract traces. The corresponding UV and mass spectra traces are shown in Fig. 4, which were recorded using the same column, LC conditions and mobile phase, but using Agilent HPLC with a Bruker microTOF MS.

It is clear from Fig. 2 that components of extract that can be quantitatively determined by the optimized HPLC-ELSD method are: artemisinin itself, deoxyartemisinin, artemisitene and artemisinic acid. Comparison of UV and ELSD traces shown in Fig. 3 demonstrates the point that due to low absorbance of artemisinin in UV, it is very easy to lose its peak amongst the noise. The peak of artemisinin is at retention time of 7.2 min in the UV trace in Fig. 3. Therefore, without the support of an additional method, the use of UV-only detection of artemisinin in extracts is not ideal.

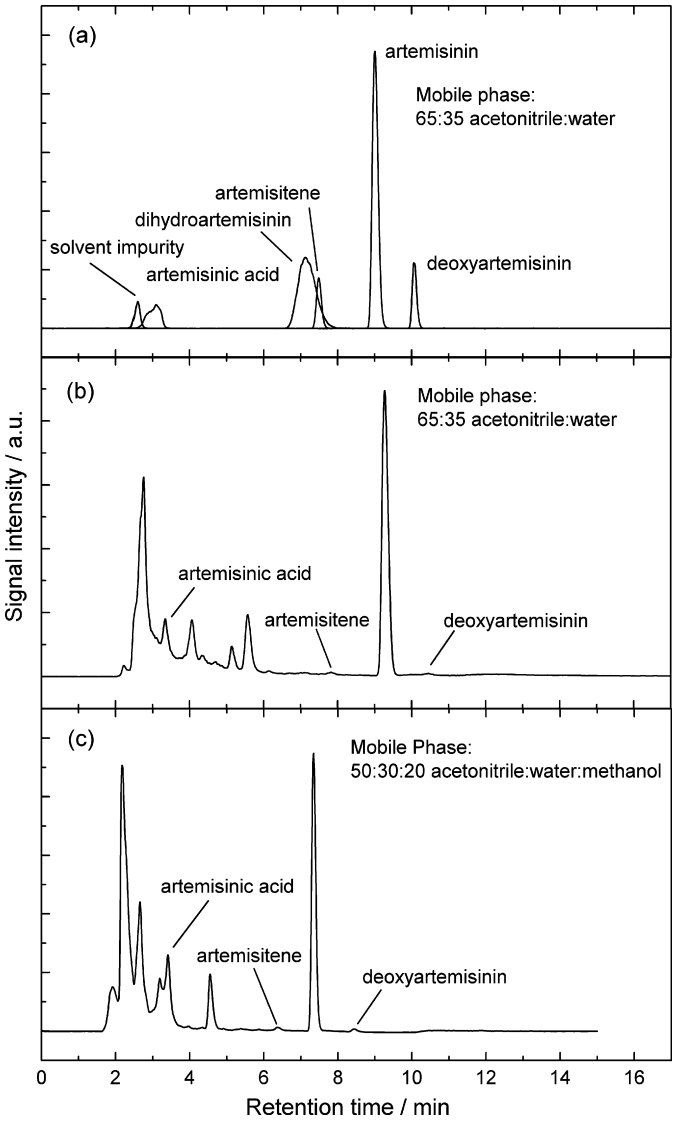


Fig. 2. Comparison of HPLC-ELSD traces obtained using two different mobile phase systems: (a) standards dissolved in acetonitrile; (b) acetonitrile:water 65:35% (v/v); (c) acetonitrile:water:methanol 50:30:20% (v/v). The concentrations of standards used: artemisinin: 1.85 mg mL^{-1}; artemisinic acid: 0.60 mg mL^{-1}, artemisitene: 0.89 mg mL^{-1}, dihydroartemisinin: 1.70 mg mL^{-1}, deoxyartemisinin: 1.42 mg mL^{-1}.

In order to attempt to identify other peaks observed in the extracts in Figs. 2 and 3, the LC method was transferred

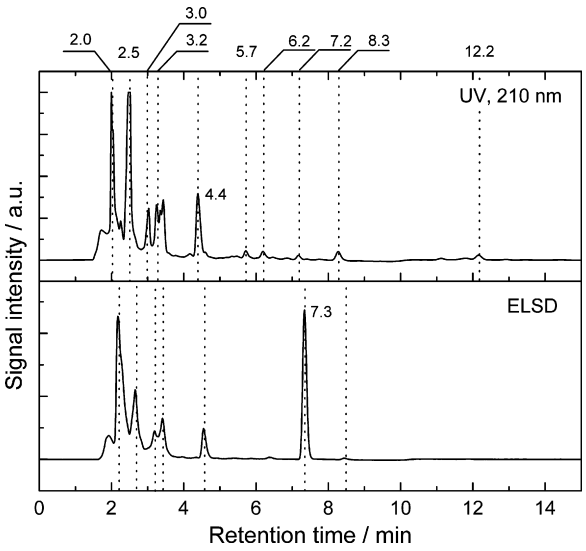


Fig. 3. An LC trace of *A. annua* ethyl acetate extract with UV and ELSD detectors in series, Shimadzu Prominence instrument. Mobile phase acetonitrile:water:methanol (50:30:20%, v/v), Betasil C18 5 μm 250 mm × 4.6 mm at column flow-rate 1.0 mL min^{-1}.

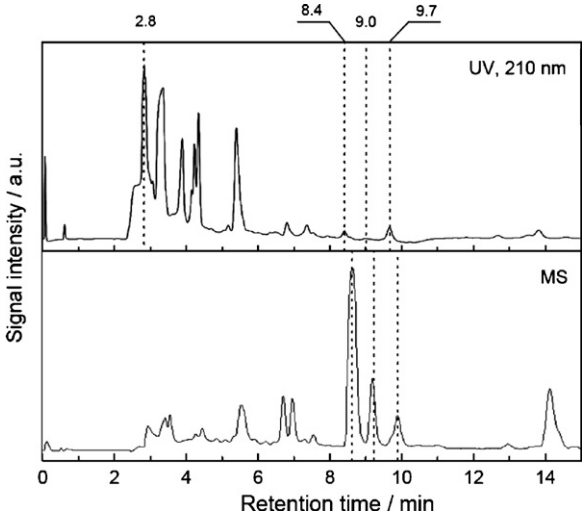


Fig. 4. An LC-MS trace of *A. annua* ethyl acetate extract with UV and time of flight (TOF) detectors, Agilent–Bruker instrument. Mobile phase acetonitrile:water:methanol (50:30:20%, v/v), Betasil C18 5 μm 250 mm × 4.6 mm at column flow-rate 1.0 mL min^{-1}. The MS was set in positive mode.

onto an LC-MS instrument. The UV traces obtained on HPLC and LC-MS instruments were very similar, although there is a noticeable shift in retention times: the peak at 2.0 min obtained on the Shimadzu HPLC instrument appears at 2.8 min on the Agilent LC, see Fig. 4 for the latter trace. The artemisinin peak shifts quite considerably from 7.2 to 8.4 min correspondingly. However, given the visual similarity of the traces, it is relatively straightforward to find the correspondence between the peaks/compounds obtained with the two different instruments.

Based on the traces obtained with UV, ELSD and MS detectors, the last two clearly give a much stronger signal of artemisinin than the UV detector. However, most significantly, the MS detects a compound at *ca*. 9 min retention times (retention time corresponds to UV trace in Fig. 4), which was not detected either by UV at 210 nm or by ELSD.

Table 8
Identification of compounds in *Artemisia annua* extracts. HPLC-UV and HPLC-ELSD analysis.

	Retention time (min), Fig. 3	Retention time in MS (min), Fig. 4	Compound
1	2.0		Solvent
2	3.2–3.4	3.3–3.6	Artemisinic acid (**3**)
3	5.7	6.8–6.9	Arteannuin-B (**2**)
4	6.2	6.9–7.1	Artemisitene (**4**)
5	7.3	8.6	Artemisinin (**1**)
6	8.3	9.9	Deoxyartemisinin (**5**)

Retention times are given according to UV trace in Fig. 3 and MS trace in Fig. 4.

Retention times of the key compounds **1**, **3**–**6** were identified using standards and confirmed by MS. The only compound which was identified only by MS was arteannuin-B (**2**). Table 8 gives retention times of these compounds, corresponding to the HPLC-UV trace in Fig. 3 and the MS trace in Fig. 4.

4. Conclusions

Within the context of an increasing number of suppliers of artemisinin and the increasing uncertainty over the quality of materials, the amount of artemisinin in particular biomass and the efficiencies of different extraction procedures, this paper systematically addressed optimization of HPLC conditions, selection of best detection techniques and the identification of the key impurities. This work is intended to contribute to standardization of procedures and ultimately to the development of a network of qualified laboratories capable of providing accurate and consistent quantification of this important natural product.

UV detection of artemisinin is certainly a viable option, however only for quantification of bulk purified artemisinin to the level of current monograph standard. The method has better accuracy and better limits of quantification in comparison to HPLC-ELSD and HPLC-RI. However, the ELSD detection method is better performing in the case of quantification of artemisinin in extracts, due to the simpler identification of the artemisinin peak. The HPLC-RI method has been extensively validated and showed a similar limit of quantification, in terms of concentration, to that of HPLC-ELSD method. However, a larger sample loop is required for the HPLC-RI method, due to the lower absolute sensitivity of this detector. Although detection of some key impurities, such as deoxyartemisinin, is possible with the HPLC-ELSD method, the accuracy of HPLC method is insufficient to improve on the current monograph purity standard of 100 ± 2%.

Clear separation of deoxyartemisinin and its quantification is possible. It was shown in this study that both UV and ELSD detectors miss some of the compounds in the extracts, which can be picked up by mass spectrometry. The importance and persistence of these compounds in consecutive processing of artemisinin is yet to be determined.

Overall, the HPLC-ELSD method appear to be the most robust for routine quantification of artemisinin in plant extracts, either for the purpose of quantification of artemisinin content in leaf, or for optimization of extraction/purification protocols. The HPLC-UV method is not recommended for the analysis of extracts, but only for the analysis of the purity of bulk artemisinin. Analysis of impurities in artemisinin beyond the level set by the current monograph requires the use of MS detection, due to its much higher sensitivity.

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