BIONIQS PROJECT REPORT

A Validated Assay for the Detection of Artemisinin

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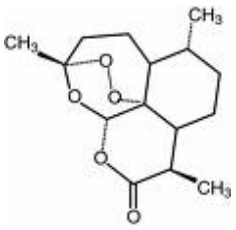
1. Summary

This document describes a validated analytical procedure, using High Performance Liquid Chromatography (HPLC) with Refractive Index (RI) detection for the quantification of artemisinin (**1**) from *Artemisia annua*. The experimental protocol followed in the development and validation of this assay was in accordance with the ICH harmonized guidelines Q2 (R1, ex A,B) text on the validation of analytic procedures, 27th October 1994.



Artemisinin (**1**)

The first section of the following report constitutes a validated assay method suitable for transfer to analytical laboratories. Subsequent sections detail the specificity, linearity, range, accuracy, precision, detection and quantitation limit, robustness and system suitability experiments which have been performed to validate the described methodology.

2. Method

2.1 Validated assay protocol

The following section constitutes a work instruction for the performance of a validated assay for the detection and quantification of artemisinin [CAS 63968-64-9].

Determine the quantity of artemisinin by high performance liquid chromatography (HPLC) using a 250x4.6mm column packed with C18 5μm stationary phase, such as Phenomenex Gemini 5μ C18 110Å, equipped with an appropriate guard column. As the mobile phase, use a mixture of 6 volumes HPLC grade acetonitrile and 4 volumes HPLC grade water.

Prepare solutions in the mobile phase. Use a standard of 4mg/mL artemisinin, or *Artemisia annua* extract prepared from 3g dried plant tissue extracted with 80mL chloroform for 10 minutes with stirring at room temperature, stripped of solvent *in vacuo* and redissolved in 5mL mobile phase. Then add an equal volume of internal standard (β-artemether prepared in mobile phase at 2.5mg/mL). Filter the sample through a 0.2μm syringe filter before injecting onto the column.

Operate with a flow rate of 1mL per minute. To detect, use a refractive index detector such as a Shodex RI-101. Prepare 200μL samples pre-mixed in a 1:1 ratio with the internal standard, in 0.3mL polypropylene HPLC vials with PTFE-lined silicone septa. Inject 25μL of the sample. The assay is not valid unless the internal standard peak has an area of 10+/-1

μRIU.min. The artemisinin peak elutes at 9-9.5 minutes, the internal standard elutes at 17-17.5 minutes. Obtain the area of the artemisinin peak in each sample, checking the automated integration by eye to ensure that it runs from baseline to baseline.

Using a set of standard solutions covering a range from 0.05mg/mL to 20mg/mL artemisinin dissolved in the mobile phase, calibrate the peak area for the quantification of artemisinin. The calibration curve should be linear in this range. Use this curve to quantify the level of artemisinin present in the sample.

When numerous analyses are to be performed in succession using the same column, an injection of neat mobile phase should be included after every sixth analyte injection and after the final sample injection of the day, to avoid the precipitation of analyte affecting the reproducibility of the assay.

3. Validation Methodology

3.1 Specificity

Two separate room temperature extractions were performed upon dried *A. annua* leaf (Mediplant, 3.0g) using chloroform (80mL). These samples (the "reference analyte extract" or RAE) were stored at 4°C and analysed by HPLC as described above. A concentrated (40mg/mL) artemisinin standard was also prepared (the "reference analyte" or RA), in order to confirm which of the RI peaks detected in the chloroform extracts corresponded to artemisinin in terms of retention time (9-9.5 minutes).

The concentrated standard RA was analysed using a run time of 20 minutes, with post-column fractions being collected on a 1-minute time-lapse basis. These fractions were re-injected independently, to confirm the elution time. The RAEs were then analysed using fraction collection under the same basis, with fractions eluting at 9-9.5 minutes being collected and analysed by LC-MS at the Department of Biology, University of York. These analyses confirmed the purity and identity of the fraction eluting at 9-9.5 minutes in both RAEs to be pure artemisinin.

A second series of extractions were performed using chloroform under identical conditions, using dried *Melissa officinalis* (Lemon Balm) leaf rather than *A. annua*. Since *M. officinalis* is known to contain no artemisinin, this sample was used as a "reference blank extract" (RBE). Analysis by HPLC according to the above method showed no peak eluting at 9-9.5 minutes in the RBE.

Given the confirmation of purity of the RAE 9-9.5 minute sample by LC-MS, the preparative extraction and analysis of arteannuin B, dihydroartemisinic acid and artemisinic acid was not considered necessary. The results from the above described work adequately validated the specificity of the assay.

3.2 Linearity

A standard plot of artemisinin concentration versus signal strength (in μRIU.min) was prepared in triplicate using the RA. The linearity of the curve plotted from the RAE was confirmed in triplicate using least squares regression analysis; the extractions in this case being diluted tenfold prior to 1:1 dilution with internal standard, hence the final dilution

factor of the RAE was 1 in 20. Samples at lower dilutions also gave a linear response (see Section 3.4 below).

3.3 Range

From the linearity, accuracy and precision results presented in the following section, the range over which the assay gave a direct linear proportionality to the artemisinin concentration was confirmed as between 0.025-20mg/mL artemisinin.

3.4 Accuracy

The accuracy of the assay was validated by spiking pure artemisinin into both *M. officinalis* (RBE) and *A. annua* (RAE) extracts, in order to determine the relationship between amount of artemisinin added and RI response. The spiked analytes were analysed alongside the RBE, RAE and various concentrations of the RA. The experiments were all performed in triplicate, using three samples of each analyte.

Results are presented in the following section. For each replicate, the following results are shown:

a.) the standard RA curve
b.) the plot of detected artemisinin (as peak area checked against the standard curve) versus amount of artemisinin added. This is fitted to a linear plot for each dilution of RAE, showing a direct correlation between the amount of artemisinin added and the amount detected. The intercept of this line gives the contribution of the RAE to the total amount of artemisinin detected. The RBE plots have not been shown as they overlay the zero *A. annua* extract – *i.e.* the only artemisinin contained in the RBE samples was that arising from the spiking process and the response from these increased in a linear fashion proportional to the amount of artemisinin added.
c.) The plot of the intercept of plot b.) above versus the dilution factor. These plots are all linear, hence the accuracy of the measurement of the total amount of artemisinin present is validated, regardless of either the amount of artemisinin added within the specified range or the dilution factor applied to the extract. The variation between the triplicate samples was minimal, confirming the accuracy of the assay.

Statistical analysis of the results to substantiate the accuracy is also shown below. Given the specificity already demonstrated, no further analysis of the spiked samples was considered necessary.

3.5 Precision

The ICH harmonized tripartite guidelines state that:

"the precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability (the intra-assay precision under the same operating conditions over a short interval of time), intermediate precision (variations within a laboratory: different days, different analysts etc.) and reproducibility (precision between laboratories assessed through collaborative studies usually applied to

standardization of methodology). Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. "

We have assessed the repeatability and intermediate precision of the assay in the following way. Three RA and three RAE samples of varying concentrations (0, 1, 3, 6 and 10mg/mL after dilution with internal standard) were prepared and each sample was analysed according to the assay protocol three times in a single day, in order to assess the reproducibility of the method. The concentration of artemisinin present in each sample was assessed by means of a standard curve prepared on the same day. This entire experiment was repeated on the two following days by different operators. By comparing the results obtained on the same day for each individual sample, repeatability values (expressed as relative standard deviation %) were obtained and are shown below. The net repeatability values are mean averages of the daily repeatability values. The intermediate precision values (expressed identically) were obtained by comparison of all of the individual results obtained for each sample over the 3 days of the assay.

Assays performed on the same day gave values varying by up to 6%, hence this represents the maximum variation and should be quoted as the +/- precision value for the assay when performed under these conditions. Assays performed on the same sample after two days gave consistently lower values, evidencing the solution-phase degradation of artemisinin in the mobile phase over time. As expected, this effect was much more pronounced at lower concentrations of artemisinin. Samples should not therefore be stored in mobile phase for longer than 12 hours prior to analysis.

3.6 Detection and Quantitation Limits

A similar methodology to that described in Section 3.4 above was employed to assess the detection and quantitation limits of the assay. Low levels of pure artemisinin were added to RAE and RBE samples, which were then progressively diluted in mobile phase to determine the maximum dilution at which artemisinin could be reproducibly and reliably detected and quantified. Background noise was determined by measuring the peak height and integration in the RBE samples between 9-9.5 minutes (the elution time of artemisinin).

As shown in the following section, the average peak height for background noise was determined to be 0.322μRIU.min. This equates to a peak height detection limit of 0.805μRIU.min and a peak height quantitation limit of 3.22μRIU.min, using signal to noise ratios of 2.5:1 and 10:1 respectively.

Peaks greater than the **detection limit** were observed in spiked RBE samples containing **0.025mg/mL** artemisinin (peak height was 0.82+/-0.2) and peaks greater than the **quantitation limit** were detected in spiked RBE samples containing **0.1mg/mL** artemisinin (peak height was 3.49+/-0.46). These concentration values thus represent the working detection and quantitation limits of the assay. For the RAE, these values equate to dilution factors of 1 in 80 for detection limit and 1 in 16 for quantitation limit.

3.7 Robustness and System Suitability

Deliberate variations made to assess the robustness and suitability of the assay in the field were confined to the detection and quantification of artemisinin, as this protocol does not represent a validated method for extraction but solely for analysis. The formulation of the mobile phase eliminates the need for any buffers, hence pH variations will be negligible if the assay method is followed and HPLC grade water is employed. The effect of prolonged storage in the mobile phase is referred to in Section 3.5 above.

The optimal mobile phase was established by using the existing International Pharmacopoeia monograph method and varying the mobile phase from 55% to 65% acetonitrile in water. Optimal separation was judged to occur at 60%, hence this formulation was adopted for the assay.

Flow rates of between 0.5-2mL/minute were evaluated, with the optimal balance between peak resolution and minimal run time being achieved at a flow rate of 1mL/minute.

4. Materials and Instrumentation

The described assay was developed using a Dionex Ultimate 3000 HPLC system running a Phenomenex Gemini 5µ C18 110Å 250x4.6mm column and a Shodex RI-101 refractive index detector. HPLC grade solvents were purchased from Fisher Scientific Limited, Loughborough, UK. HPLC vials and septa were purchased from Dionex Incorporated, Sunnyvale, California, USA. Artemisinin was supplied by Medicines for Malaria Venture (MMV), Geneva, Switzerland. β-Artemether was purchased from Apin Chemicals Limited, Abingdon, UK. *Artemisia annua* was kindly donated by Dr. Xavier Simonnet of Mediplant, Conthey, Switzerland. *Melissa officinalis* was purchased from Cotswold Health Products Limited, Wotton-under-Edge, UK. LC-MS analyses were performed at CNAP, Department of Biology, University of York, UK.

5. Results

5.1 Specificity

<u>Establishing conditions for collection of 'artemisinin' peak</u>

- The 'artemisinin' peak routinely eluted at 9-9.5 minutes under the standard conditions. This peak could be collected manually (soon after leaving the RI detector), or using a fraction collector.
- The tubing leading the sample to the fraction collector had an unknown volume, so it was necessary to work out which fraction(s) contain the target peak. To do this, 30mg/mL artemisinin was injected, and the fraction collector was started at the same time, collecting 1 minute (= 1 ml) fractions (HPLC sequence 07June07, sample 1).
- Samples of fractions 10 to 24 were then injected onto the HPLC, to look for the presence of artemisinin (HPLC sequence 07June07, samples 3 to 17). A diluted standard was also injected, to compare with the actual samples (HPLC sequence 07June07, sample 2).
- The peak areas and retention times were as follows:

name	RT of artemisinin peak (mins)	peak area (µIU*min)	comments
standard (concentrated)	9.239	104.737	this is the major peak, which spans 1 min (= 1 ml)
diluted standard	9.61	0.967	sloping baseline
tube 23	none	-	
tube 10	none	-	
tube 11	none	-	
tube 12	9.693	0.785	
tube 13	9.72	1.173	
tube 14	9.7	0.618	
tube 15	9.73	0.172	
tube 16	small peak	too small	
tube 17	none	-	
tube 18	none	-	
tube 19	none	-	
tube 20	none	-	
tube 21	none	-	
tube 22	none	-	
tube 24	none	-	

- The standard was 30mg/mL artemisinin and the diluted standard was 25µL made up to 1mL (to represent 25µL injection being diluted into a 1mL fraction) = 40-fold dilution.
- A selection of the fractions collected throughout the HPLC run of the concentrated standard were re-run on the HPLC to locate the artemisinin. The artemisinin was found in tubes 12 to 15, with the highest concentration in tube 13. This meant that a peak eluting at 9.239 minutes, and spanning the minute from 9:00 to 10:00 mins (= 1ml), was now spread over 4 x 1 ml fractions

Collecting peaks for analysis

Peaks were collected manually and using the fraction collector, as follows:

Sample ID	Sample injected	Fraction collected
PEAK_EX_1	Plant extract (HPLC 06June07, no. 12)	By hand, with tubing
P1_EX_2	Plant extract (HPLC 06June07, no. 13)	By hand, minimal tubing
ART_ST_PEAK	Artemisinin (HPLC 06June07, no. 13)	By hand, minimal tubing
5G_L_STD	5mg/mL artemisinin (HPLC 08June07, no. 9)	Fraction 13
BLANK_SAMPLE	5mg/mL artemisinin (HPLC 08June07, no. 9)	Fraction 20 (expect no artemisinin)
AM_EXT_1	Plant extract (HPLC 08June07, no. 7)	Fraction 13
AM_EXT_2	Plant extract (HPLC 08June07, no. 8)	Fraction 13

Summary of LC-MS analysis

- Samples collected by hand contain only low levels of artemisinin, which is probably because it was not possible to know exactly what time to perform the collection because the volume of tubing from the detector was unknown (data not shown in this summary).
- Fraction 13 from the artemisinin standard contained nearly-pure artemisinin (figure 1)
- Fraction 20 from the artemisinin standard contained no artemisinin, as expected (figure 2)
- Fraction 13 from both of the Artemisinin plant extracts contains nearly-pure artemisinin (e.g. figure 3, for plant extract 1). There is considerable variation in the overall amount of artemisinin detected in these two equivalent fractions, but importantly both are of comparable purity to the artemisinin standard.

Overall conclusion: The peak that appears to be artemisinin in the HPLC-RI validated assay is confirmed to be artemisinin using a well-established LC-MS assay

Table 1: LC-MS data summary

Filename	Exp Amt	Calc Amt	Units	%Diff	Level	%RSD-AMT	Peak Status
Blank_1	NA	NF	ng	NF	NA	NF	Not Found
11	1.953	1.797	ng	-8%	11	31.9%	
10	3.906	4.333	ng	11%	10	22.4%	
7	31.250	34.628	ng	11%	7	6.1%	
6	62.500	64.414	ng	3%	6	1.9%	
5	125.000	119.924	ng	-4%	5	1.9%	
3	500.000	535.286	ng	7%	3	11.3%	Response High
Blank_2	NA	NF	ng	NF	NA	NF	Not Found
P1_EX_2	NA	14.127	ng	NA	NA	NA	
PEAK_EX_P1	NA	0.822	ng	NA	NA	NA	
ART_ST_PEAK	NA	0.056	ng	NA	NA	NA	
AM_EXT_2	NA	24.580	ng	NA	NA	NA	
BLANK_SAMPLE	NA	NF	ng	NF	NA	NF	Not Found
5G_L_STD	NA	66.588	ng	NA	NA	NA	
AM_EXT_1	NA	100.233	ng	NA	NA	NA	

Standards for the LC-MS technique are in grey. Samples from the HPLC-RI validated assay are in black.

Figure 1: 5mg/mL artemisinin standard, fraction 13

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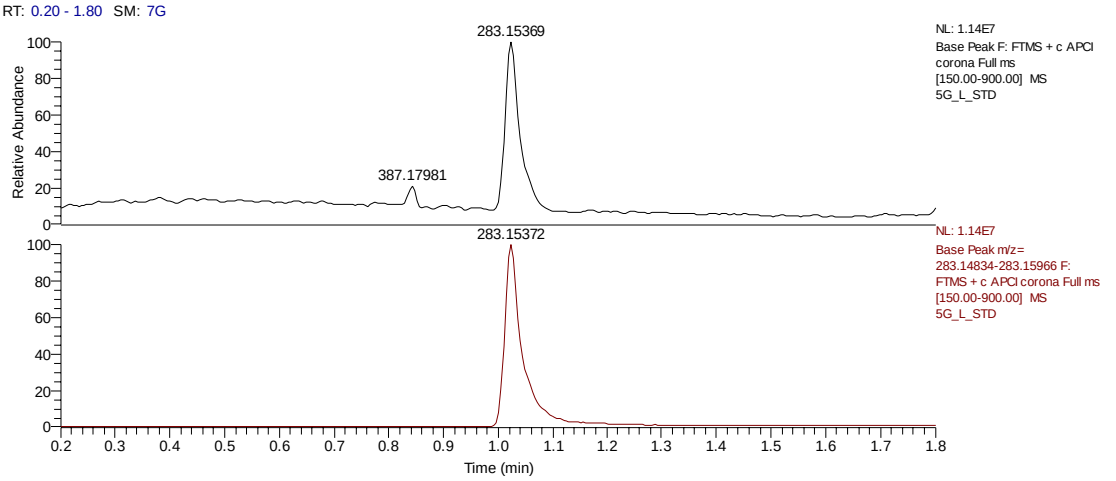

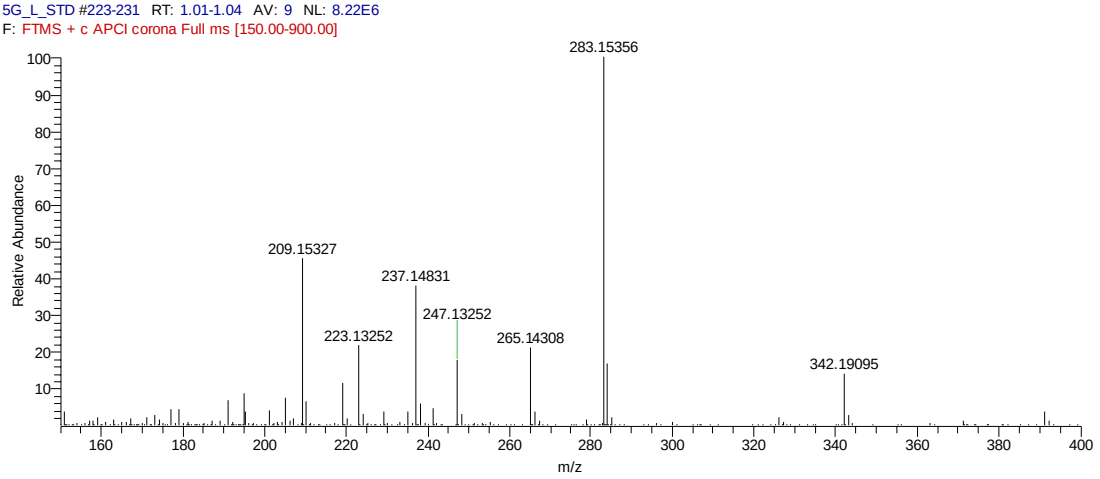

Figure 2: 5mg/mL artemisinin standard, fraction 20 (should contain no artemisinin)

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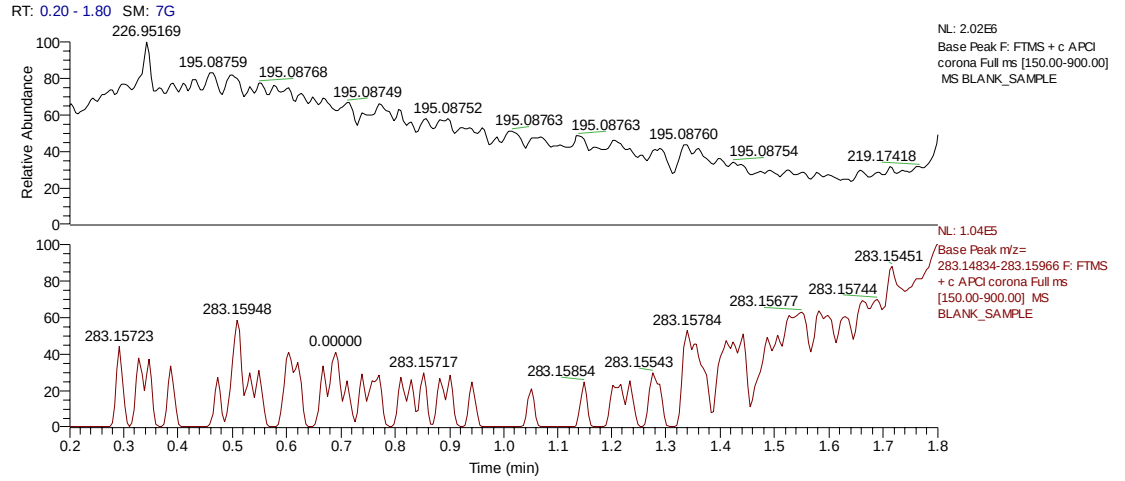


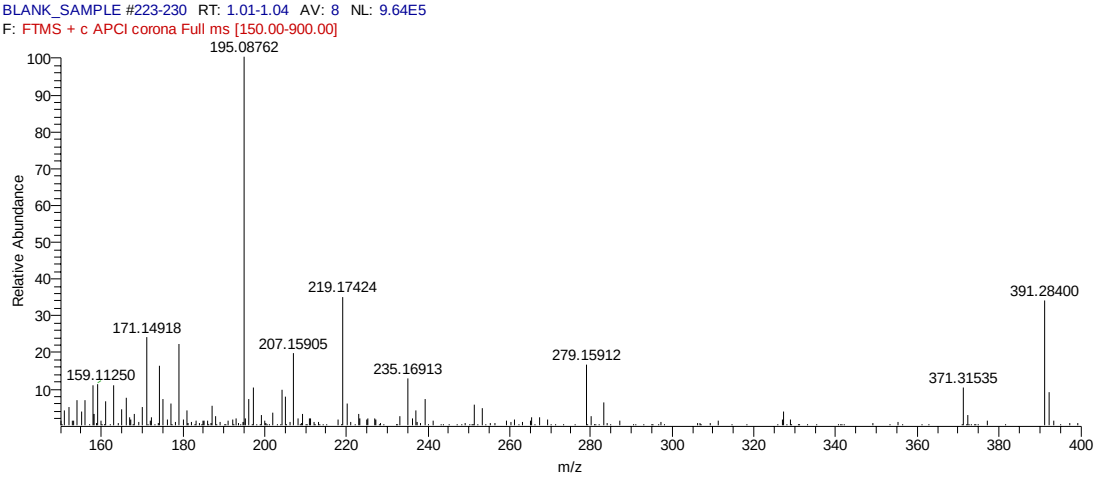
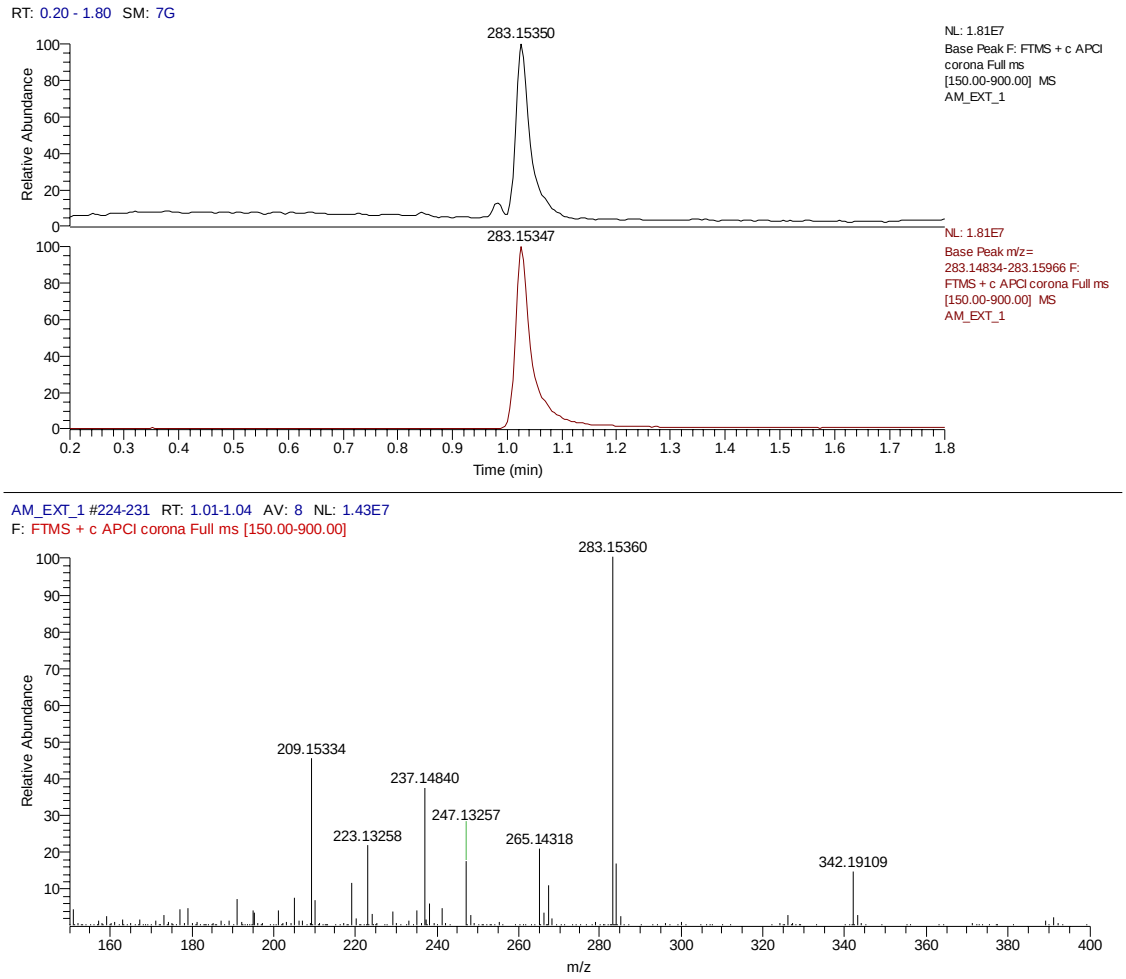


Figure 3: Artemisia plant extract # 1, fraction 13

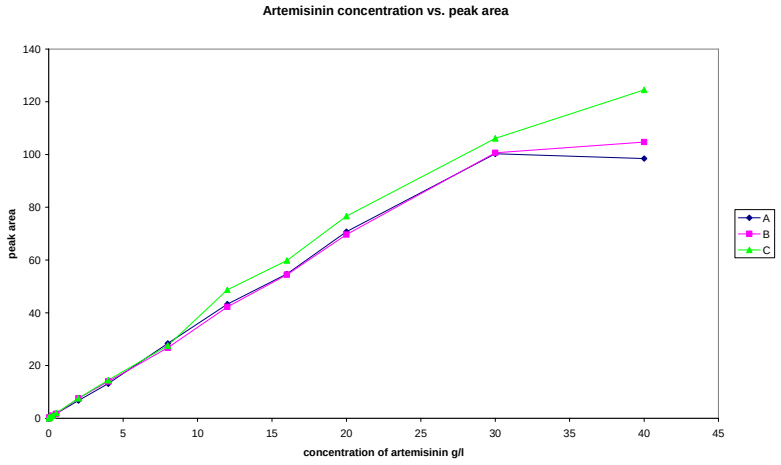


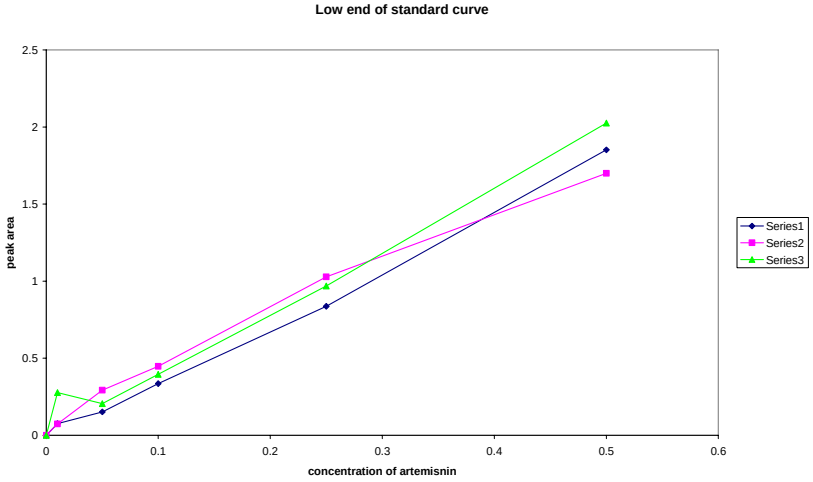
5.2 Linearity

A standard curve of artemisinin was prepared in triplicate, summarized below:

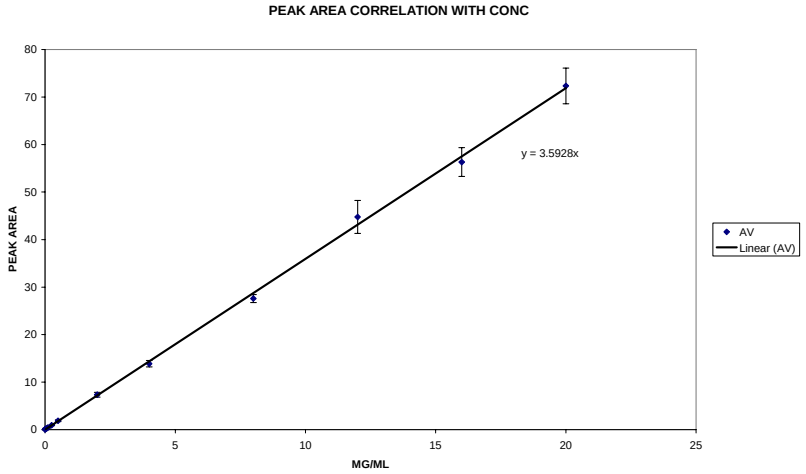
peak area

	A	B	C	AV	SD	retention time		
40	98.509	104.696	124.551	109.252	13.60567	A	B	
30	100.249	100.677	106.125	102.3503	3.275954	40	9.5	9.49
20	70.743	69.657	76.649	72.34967	3.762718	30	9.48	9.52
16	54.689	54.441	59.825	56.31833	3.039393	20	9.58	9.57
12	43.293	42.254	48.729	44.75867	3.477433	16	9.63	9.61
8	28.438	26.747	27.557	27.58067	0.845748	12	9.64	9.68
4	13.158	13.982	14.497	13.879	0.675416	8	9.72	9.71
2	6.754	7.626	7.628	7.336	0.504028	4	9.81	9.77
0.5	1.851	1.7	2.025	1.858667	0.162636	2	9.85	9.82
0.25	0.837	1.028	0.968	0.944333	0.097675	0.5	9.85	9.84
0.1	0.335	0.447	0.395	0.392333	0.056048	0.25	9.89	9.85
0.05	0.152	0.293	0.206	0.217	0.071141	0.1	9.88	9.83
0.01	0.076	0.073	0.277	0.142	0.116923	0.05	9.89	9.88
0	0	0	0	0	0	0.01	9.86	9.86





The average peak areas versus concentration were plotted as below:



The graph shows that the correlation was linear up to 20mg/mL, hence the detection was linear to this limit.

The linearity of *Artemisia* extract (RAE) was also checked from triplicate extractions. These were diluted down to 1 in 2o,(by the time they were diluted 1 in 2 with internal standard)

The peak areas are shown below:

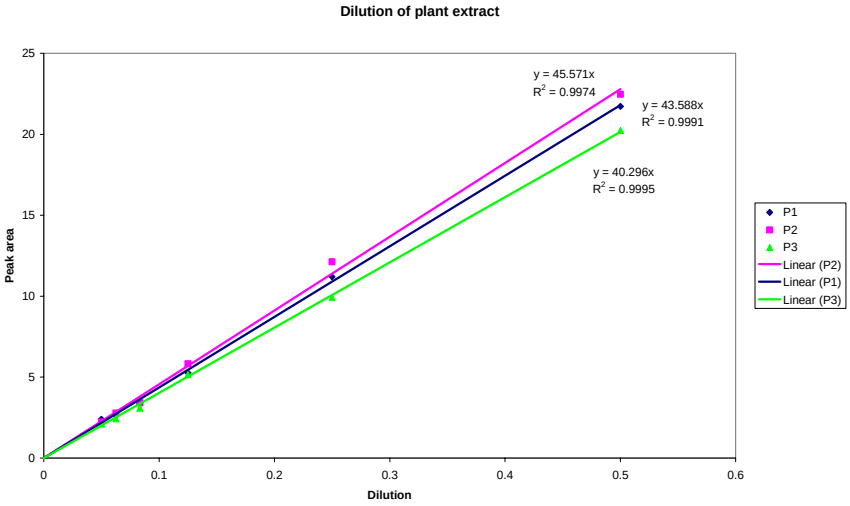
Art

	P1	P2	P3
0.5	21.723	22.465	20.231
0.25	11.198	12.123	9.939
0.125	5.268	5.828	5.162
0.0833	3.363	3.411	3.088
0.0625	2.631	2.781	2.428
0.05	2.397	2.231	2.111

Nab

	P1	P2	P3
0.5	14.027	13.575	13.48
0.25	13.715	14.074	13.813
0.125	14.311	14.077	14.282
0.0833	16.401	16.323	16.608
0.0625	14.704	14.3	14.359
0.05	14.552	14.621	14.556

Different plant extracts start with slightly different amounts of artemisinin, but each one has a linear relationship with dilution down to at least 1 in 20 dilution.



The accuracy experiments showed the retention of accuracy to dilutions of 1 in 32 – see below.

5.3 Accuracy

Samples were numbered as follows:

	T0	T0.125	T0.5	T2	T8
A-ve	**1 A0-T0**	**2 A0-T0.125**	**3 A0-T0.5**	**4 A0-T2**	**5 A0-T8**
A2	**6 A2-T0**	**7 A2-T0.125**	**8 A2-T0.5**	**9 A2-T2**	**10 A2-T8**
A8	**11 A8-T0**	**12 A8-T0.125**	**13 A8-T0.5**	**14 A8-T2**	**15 A8-T8**
A32	**16 A32-T0**	**17 A32-T0.125**	**18 A32-T0.5**	**19 A32-T2**	**20 A32-T8**
L-ve	**21 L0-T0**	**22 L0-T0.125**	**23 L0-T0.5**	**24 L0-T2**	**25 L0-T8**
L2	**26 L2-T0**	**27 L2-T0.125**	**28 L2-T0.5**	**29 L2-T2**	**30 L2-T8**
L8	**31 L8-T0**	**32 L8-T0.125**	**33 L8-T0.5**	**34 L8-T2**	**35 L8-T8**
L32	**36 L32-T0**	**37 L32-T0.125**	**38 L32-T0.5**	**39 L32-T2**	**40 L32-T8**

In the following table, “NAB area” refers to the internal standard peak.

			Nab area				Nab area				
1	0		15.084	1	0		13.149			1	0
2	1.349		15.669	2	1.15		14.105			2	1.152
3	4.592		14.789	3	4.568		14.343			3	4.387
4	16.887		14.376	4	17.4		13.893			4	17.451
5	66.379		14.253	5	69.491		14.113			5	69.113
6	15.763		14.952	6			19.354		5.705	6	16.366
7	18.378		13.941	7	17.677		13.961			7	
8	21.043		14.417	8			1.879		0.175	8	
9	33.246		13.975	9	34.001		14.044			9	34.47
10	81.85		13.84	10	83.269		13.496			10	
11	4.03		15.455	11			2.406		1.407	11	5.593
12	5.425		14.724	12	5.135		14.319			12	5.456
13	8.91		14.606	13	8.431		14.532			13	11.481
14	21.339		14.687	14	21.846		14.496			14	21.62
15	68.256		13.728	15	72.4			86.926	16.874	15	72.154
16	1.085		15.189	16	1.154		14.223			16	1.273
17	2.479		15.778	17			2.167		0.947	17	
18	5.784		15.031	18	5.464		14.501			18	5.519
19	17.973		14.51	19	18.378		14.239			19	19.428
20	69.562		14.702	20	69.391		13.88			20	69.505
21	0		15.275	21	0		15.428			21	0
22	1.584		19.017	22	1.291		15.272			22	
23	4.721		15.23	23	4.854		15.506			23	
24	17.311		14.638	24	18.88		15.252			24	17.612
25	69.462		14.784	25	73.58		14.716			25	68.497
26	0		15.426	26	0		14.807			26	0
27	1.21		14.599	27			0.508		0.324	27	1.219
28	4.775		14.98	28	5.349		17.547			28	
29	16.746		14.374	29	19.849		16.247			29	17.789
30	67.327		14.508	30	78.94		15.975			30	70.621
31	0		18.801	31	0		13.535			31	0
32	1.178		14.364	32	1.357		14.422			32	1.257
33	4.797		14.918	33	4.422		13.91			33	4.584
34	16.911		14.49	34	17.562		13.968			34	18.115
35	67.203		14.204	35	68.501		13.676			35	
36	0		16.799	36	0		13.179			36	
37	1.402		16.538	37	1.182		14.462			37	1.697
38	5.026		15.583	38	4.59		14.595			38	
39	17.781		15.051	39	21.603		17.434			39	17.401
40	70.151		14.923	40	68.922		13.852			40	79.489

The areas for the three sets of standard curves are shown below with mean averages and standard deviations.

First set of data

	Art area		Av	sd
S0a	0	0	0	0
S0b	0			
S0c	0			
S0.1a	0.594	0.2	0.598	0.005292
S0.1b	0.604			
S0.1c	0.596			
S0.464a	3.23	0.928	3.247	0.020664
S0.464b	3.241			
S0.464c	3.27			
S2.15a	15.691	4.3	15.78567	0.107156
S2.15b	15.764			
S2.15c	15.902			
S5a	41.148	10	41.16067	0.043409
S5b	41.209			
S5c	41.125			
S10a	81.435	20	80.373	1.888143
S10b	81.491			
S10c	78.193			

Nab area

1.37	
50.915	
10.639	
0.321	

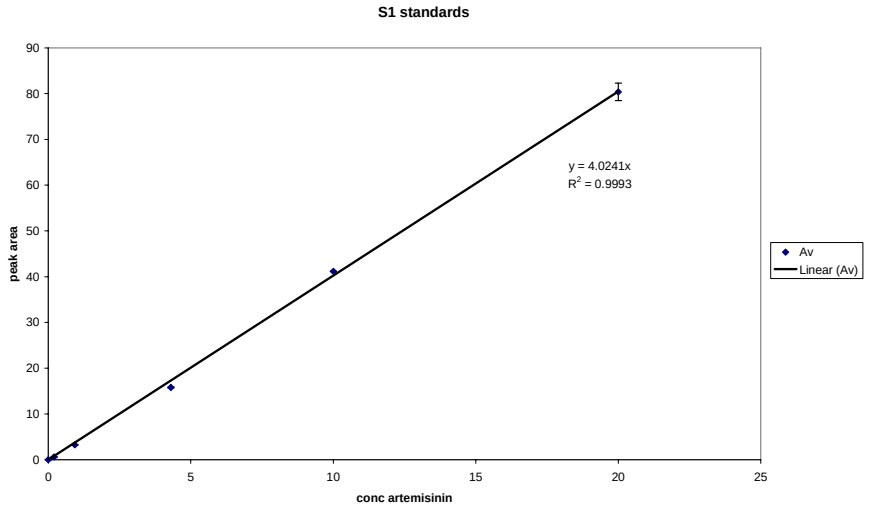
second set

	Art area		Av	sd
S0a	0	0	0	0
S0b	0			
S0c	0			
S0.1a	0.739	0.2	0.729667	0.009018
S0.1b	0.721			
S0.1c	0.729			
S0.464a	3.934	0.928	3.9695	0.050205
S0.464b	4.005			
S0.464c				
S2.15a	17.806	4.3	17.06567	1.356573
S2.15b	17.891			
S2.15c	15.5			
S5a				
S5b				
S5c				
S10a	97.799	20	81.00833	29.3781
S10b	98.14			
S10c	47.086			

third set

	Art area		Av	sd
S0a		0	0	#DIV/0!
S0b				
S0c				
S0.1a	0.136	0.2	0.593667	0.410671
S0.1b	0.93			
S0.1c	0.715			
S0.464a	4.155	0.928	3.957333	0.382897
S0.464b	4.201			
S0.464c	3.516			
S2.15a	18.456	4.3	18.056	4.3
S2.15b	18.458			
S2.15c	17.254			
S5a	40.573	10	40.66533	10
S5b	43.679			
S5c	37.744			
S10a	87.641	20		#DIV/0!
S10b				
S10c				

For the first standard curve a linear plot was obtained:



From this standard curve the amount of artemisinin in the samples was calculated, by dividing the area by 4.024:

results for firast set of samples
convert first set to artemisinn(/4.0214)

	A-ve	A2	A8	A32	L-ve	L2	L8	L32
T0	0	3.91977918	1.002138559	0.269806535	0	0	0	0
T0.25	0.335455	4.57005023	1.349032675	0.616451982	0.393893	0.30089	0.292933	0.348635
T1	1.141891	5.23275476	2.215646292	1.438305068	1.173969	1.187397	1.192868	1.249813
T4	4.199284	8.2672701	5.306360969	4.469339036	4.30472	4.164221	4.205252	4.421594
T16	16.50644	20.3536082	16.97319342	17.29795594	17.27309	16.74218	16.71134	17.44442

	A-ve	A2	A8	A32	L-ve
0	0	3.919779	1.002139	0.269807	0
0.25	0.335455	4.57005	1.349033	0.616452	0.393893
1	1.141891	5.232755	2.215646	1.438305	1.173969
4	4.199284	8.26727	5.306361	4.469339	4.30472
16	16.50644	20.35361	16.97319	17.29796	17.27309

from art and lemon balm extract subtract amount of artemisinin from extract

	A-ve	A2	A8	A32	L-ve	L2	L8	L32	Av	sd	% error
T0	0	1.8138E-07	5.58711E-07	5.35038E-07	0	0	0	0	1.59391E-07	2.47E-07	
T0.25	0.335455	0.65027123	0.346894675	0.346645982	0.393893	0.30089	0.292933	0.348635	0.376952216	0.114755	30.5
T1	1.141891	1.31297576	1.213508292	1.168499068	1.173969	1.187397	1.192868	1.249813	1.205115293	0.054082	4.51
T4	4.199284	4.3474911	4.304222969	4.199533036	4.30472	4.164221	4.205252	4.421594	4.26828981	0.08999	2.11
T16	16.50644	16.4338292	15.97105542	17.02814994	17.27309	16.74218	16.71134	17.44442	16.76381376	0.477984	2.85

	intercept
0	0.0681
0.03125	0.3103
0.125	1.1541
0.5	4.1631

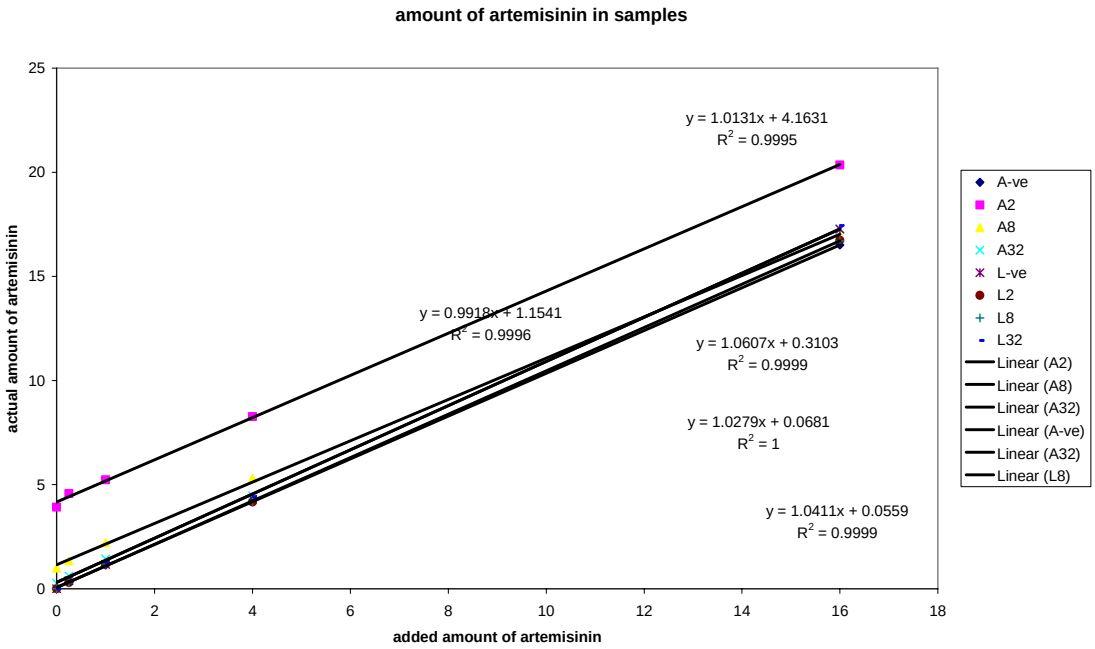
subtract amount of added artemisnin from extract to see what is from extract

	A-ve	A2	A8	A32	L-ve	L2	L8	L32
T0	0	3.91977918	1.002138559	0.269806535	0	0	0	0
T0.25	0.085455	4.32005023	1.099032675	0.366451982	0.143893	0.05089	0.042933	0.098635
T1	0.141891	4.23275476	1.215646292	0.438305068	0.173969	0.187397	0.192868	0.249813
T4	0.199284	4.2672701	1.306360969	0.469339036	0.30472	0.164221	0.205252	0.421594
T16	0.506441	4.3536082	0.973193415	1.297955936	1.273089	0.742179	0.711344	
Av	0.186614	4.2186925	1.119274382	0.568371711	0.379134	0.228938	0.230479	0.192511	0.261199949
sd									

The amount of artemisinin present in the sample was then plotted versus the amount added:

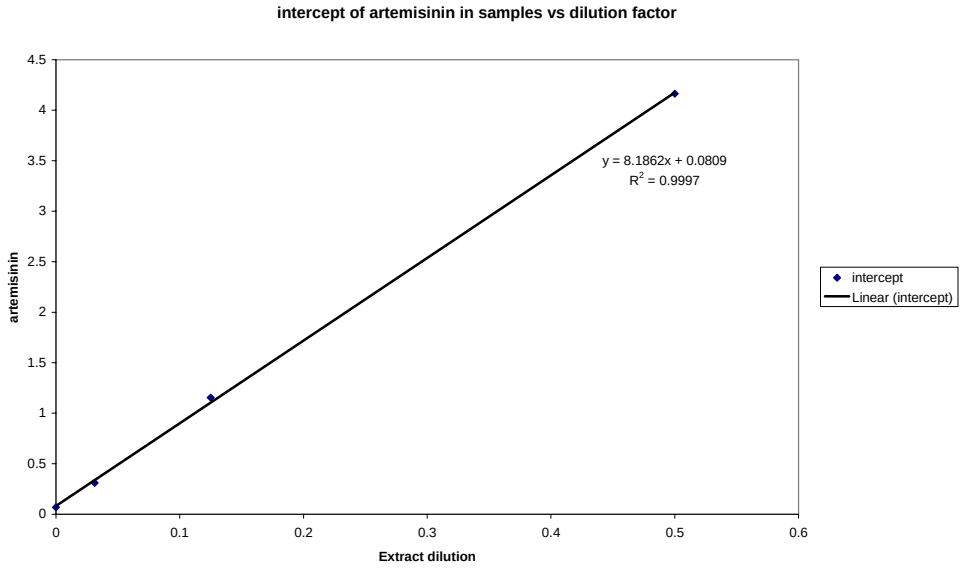
	A-ve	A2	A8	A32	L-ve	L2	L8	L32
0	0	3.919779	1.002139	0.269807	0	0	0	0
0.25	0.335455	4.57005	1.349033	0.616452	0.393893	0.30089	0.292933	0.348635
1	1.141891	5.232755	2.215646	1.438305	1.173969	1.187397	1.192868	1.249813
4	4.199284	8.26727	5.306361	4.469339	4.30472	4.164221	4.205252	4.421594
16	16.50644	20.35361	16.97319	17.29796	17.27309	16.74218	16.71134	17.44442

To give:



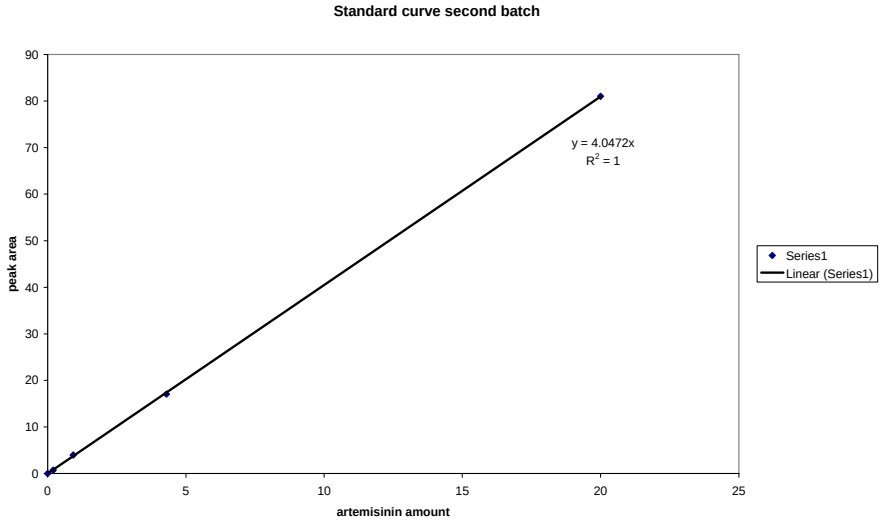
For each sample the added amount of artemisinin increased linearly with the amount added and all plots had the same gradient, showing that they all increased in an equivalent manner. The different intercepts on the y axis showed that the samples started off with different amounts of artemisinin before extra was added- *i.e.* from the *Artemisia* RAE itself.

The intercept on the y axis was plotted against the extract dilution factor to give:



The above plot was linear from 0 to 0.5 (*i.e.* two-fold dilution) showing that the system can detect the artemisinin in the extract. Since the gradients of the previous graph were around 1, the system can also detect the artemisinin added to any dilution of extract.

This procedure was repeated for the second set of results:



using the second standard curve to calculate the amount of artemisinin (dividing area by 4.047).

second set of sample- peak area

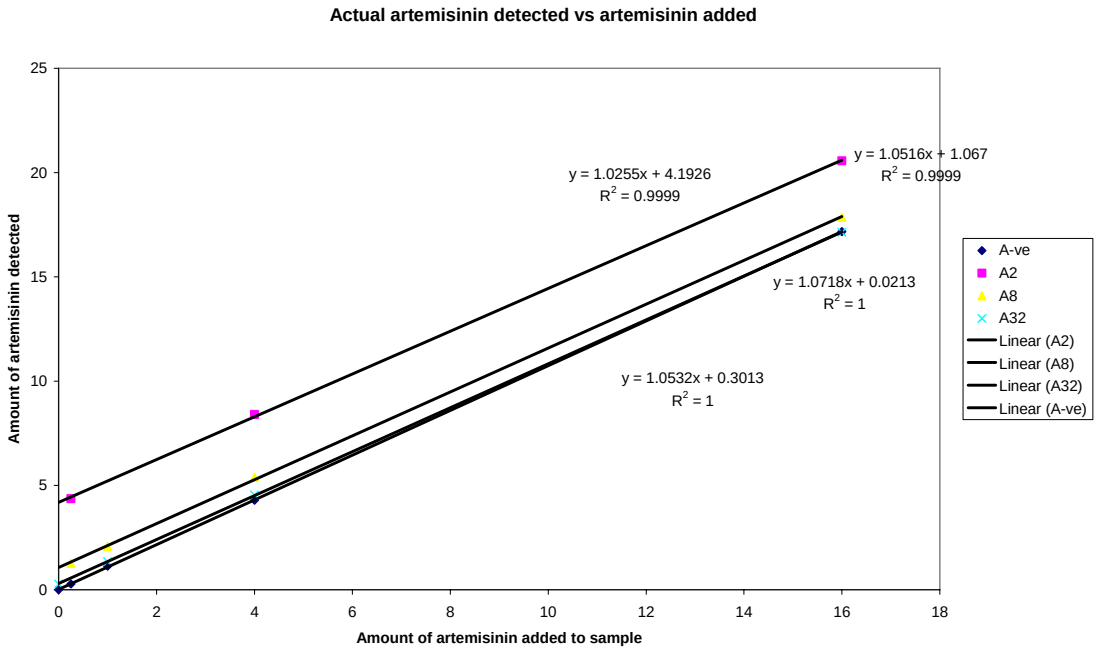
	A-ve	A2	A8	A32	L-ve	L2	L8	L32
T0	0			1.154	0	0	0	0
T0.25	1.15	17.677	5.135		1.291		1.357	1.182
T1	4.568		8.431	5.464	4.854	5.349	4.422	4.59
T4	17.4	34.001	21.846	18.378	18.88	19.849	17.562	21.603
T16	69.491	83.269	72.3	69.391	73.58	78.94	68.501	68.922

results for second set of samples
convert first set to artemisinn(/4.0472)

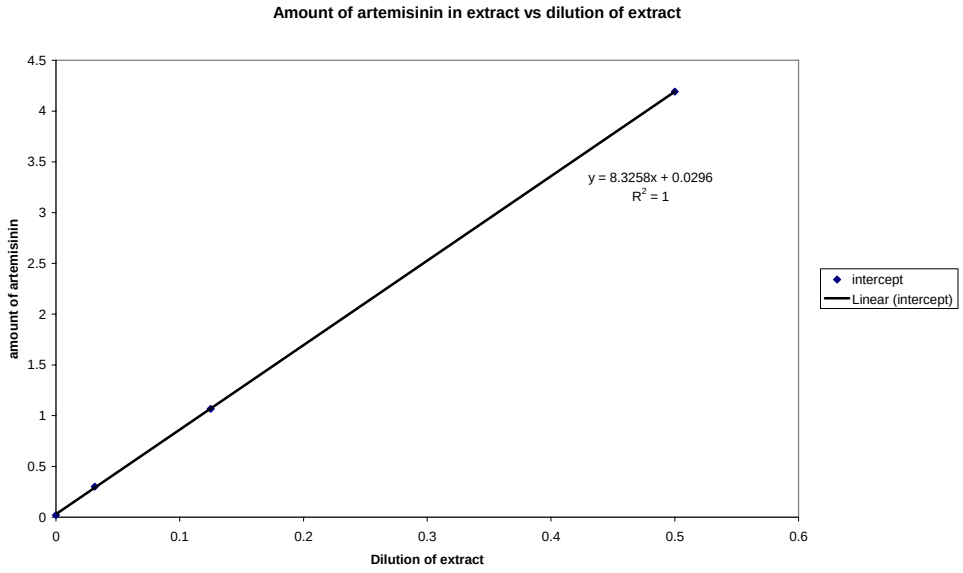
	A-ve	A2	A8	A32	L-ve	L2	L8	L32
T0	0	0	0	0.285135402	0	0	0	0
T0.25	0.284147	4.367711	1.268778	0	0.318986	0	0.335294	0.292054
T1	1.128682		2.083169	1.350069184	1.199348	1.321654	1.092607	1.134117
T4	4.299269	8.401117	5.397806	4.540917177	4.664954	4.904378	4.339296	5.337764
T16	17.17014	20.57447	17.8642	17.14543388	18.18047	19.50484	16.92553	17.02955

Actual amount of artemisinin

	A-ve	A2	A8	A32	L-ve	L2	L8
0	0			0.285135	0	0	0
0.25	0.284147	4.367711	1.268778		0.318986		0.335294
1	1.128682		2.083169	1.350069	1.199348	1.321654	1.092607
4	4.299269	8.401117	5.397806	4.540917	4.664954	4.904378	4.339296
16	17.17014	20.57447	17.8642	17.14543	18.18047	19.50484	16.92553

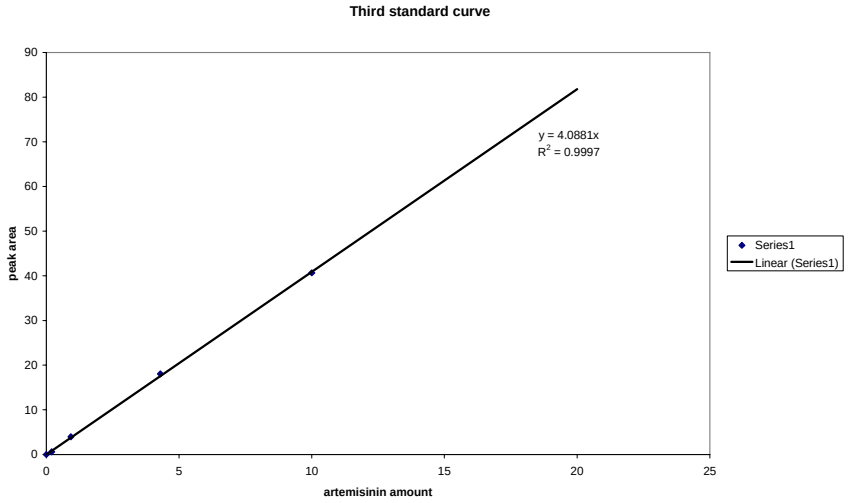


The gradients and linear increase were similar to those previously obtained.



Again the amount of artemisinin in the extract showed a linear relationship with dilution factor.

For the third data set:



Third standard curve used to convert peak area of set 3 data to artemisinin content (dividing by 4.088):

sample- peak area

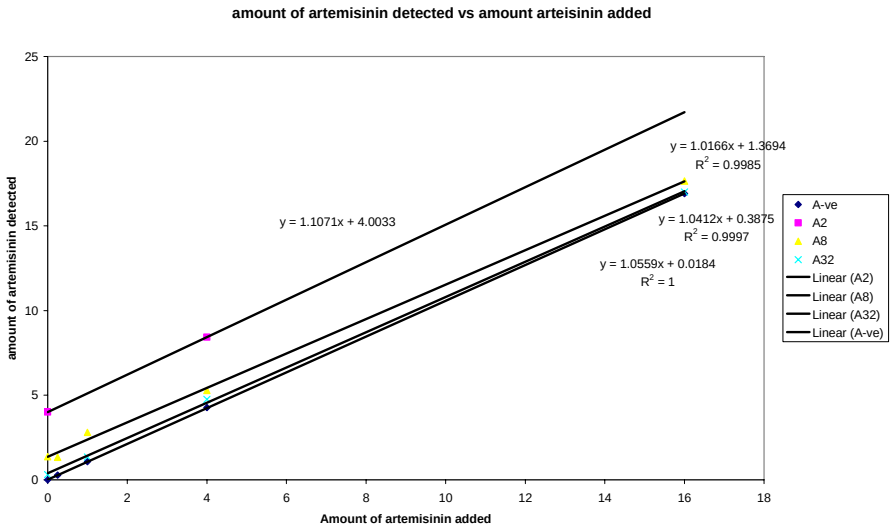
A-ve	A2	A8	A32	L-ve	L2	L8	L32
0	16.366	5.593	1.273	0	0	0	
1.152		5.456			1.219	1.257	1.697
4.387		11.481	5.519			4.584	
17.451	34.47	21.62	19.428	17.612	17.789	18.115	17.401
69.113		72.154	69.505	68.497	70.621		79.489

firast set of samples
t set to artemisinn(/4.0881)

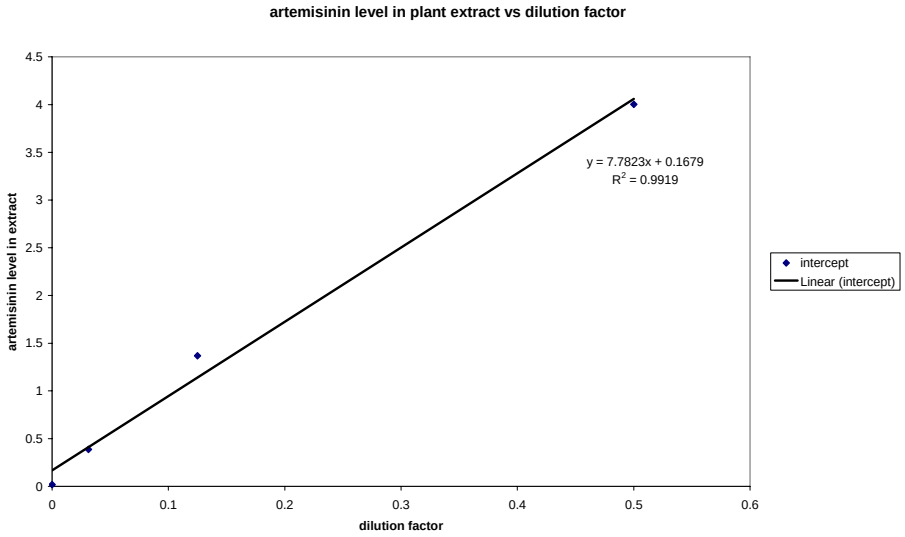
A-ve	A2	A8	A32	L-ve	L2	L8	L32
0	4.003326729	1.368117218	0.3113916	0	0	0	0
0.281793		1.334605318	0	0	0.298183	0.307478	0.415107
1.073115		2.808395098	1.3500159	0	0	1.121303	0
4.268731	8.431789829	5.28852034	4.752329933	4.308114	4.35141	4.431154	4.256501
16.9059	0	17.64976395	17.00178567	16.75522	17.27477	0	19.444

	A-ve	A2	A8	A32	L-ve	L2	L8
0	0	4.003327	1.368117	0.311392	0	0	0
0.25	0.281793		1.334605			0.298183	0.307478
1	1.073115		2.808395	1.350016			1.121303
4	4.268731	8.43179	5.28852	4.75233	4.308114	4.35141	4.431154
16	16.9059		17.64976	17.00179	16.75522	17.27477	

Plotting the amount added against amount detected again gave similar results:



Plotting the intercept against dilution also gave a linear result:



5.4 Precision

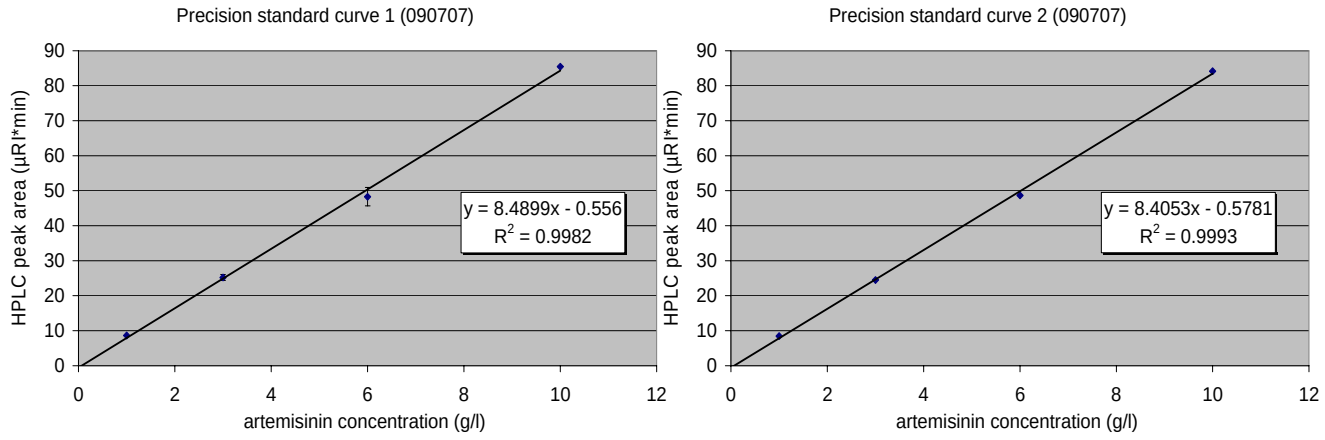
In this summary, the first two sets of precision samples are analyzed concurrently, followed by the third set. Then the data are analyzed together, and final conclusions are presented.

Analysis of data sets 1 and 2

- Peaks areas for artemisinin and the internal standard were obtained from the relevant HPLC chromatograms

Precision assay data set 1				Precision assay data set 2			
		peak area (µRIU*min)				peak area (µRIU*min)	
		Art	Stand			Art	Stand
SC	1	8.615	12.257		1	8.446	12.172
	3	25.822	13.317		3	24.31	12.911
	6	46.436	13.76		6	48.501	12.966
	10	85.441	12.36		10	84.683	12.023
A	1	70.988	12.232	A	1	67.936	12.894
	2	58.714	10.294		2	68.326	12.656
	3	68.56	12.491		3	70.793	12.344
B	1	17.17	12.528	B	1	15.895	13.184
	2	16.431	14.595		2	18.963	15.494
	3	17.659	12.218		3	16.815	14.293
C	1	5.138	14.987	C	1	4.364	12.673
	2	5.114	14.294		2	4.583	12.191
	3	4.437	12.664		3	4.633	12.219
D	1	18.577	12.861	D	1	18.672	13.095
	2	19.72	11.926		2	19.5	12.355
	3	23.105	14.051		3	6.974	1.146
E	1	4.857	12.985	E	1	5.08	12.952
	2	5.055	11.599		2	5.136	12.098
	3	19.129	28.817		3	5.717	14.105
F	1	1.352	12.314	F	1	1.244	12.751
	2	1.43	11.743		2	0.203	0.157
	3	1.55	11.371		3	0.204	0.22
SC	1	8.638	12.691		1	8.465	12.071
	3	24.613	12.976		3	24.715	12.789
	6	50.151	12.536		6	48.818	12.148
	10	85.432	12.592		10	83.65	12.547

- For each precision assay, standard curves were plotted (SC1, 3, 6 and 10), using average values from both standard curves. The standard deviations are displayed as error bars.



- The equations from the lines of best fit were used to calculate the amount of artemisinin in each sample. Note that the obliterated figures represent failed injections and were thus not included in the calculations for mean, standard deviation (StDev) and relative standard deviation (RSD):

Sample set 1			**Sample set 2**		
sample A (8 g/l)			sample A (8 g/l)		
8.427	mean =	8.284	8.151	mean =	8.280
~~6.981~~	StDev =	0.202	8.198	StDev =	0.184
8.141	RSD =	2.441 %	8.491	RSD =	2.226 %
sample B (2 g/l)			sample B (2 g/l)		
2.088	mean =	2.078	1.960	mean =	2.015
2.001	StDev =	0.073	~~2.325~~	StDev =	0.077
2.145	RSD =	3.504 %	2.069	RSD =	3.842 %
sample C (0.5 g/l)			sample C (0.5 g/l)		
0.671	mean =	0.642	0.588	mean =	0.607
0.668	StDev =	0.047	0.614	StDev =	0.017
0.588	RSD =	7.299 %	0.620	RSD =	2.803 %
sample D (2-fold dilution of extract)			sample D (2-fold dilution of extract)		
2.254	mean =	2.476	2.290	mean =	2.339
2.388	StDev =	0.277	2.389	StDev =	0.070
2.787	RSD =	11.200 %	~~0.898~~	RSD =	2.977 %
sample E (8-fold dilution of extract)			sample E (8-fold dilution of extract)		
0.638	mean =	0.649	0.673	mean =	0.701
0.661	StDev =	0.016	0.680	StDev =	0.042
~~2.319~~	RSD =	2.540 %	0.749	RSD =	5.989 %
sample F (32-fold dilution of extract)			sample F (32-fold dilution of extract)		
0.225	mean =	0.236	0.217	mean =	n/a
0.234	StDev =	0.012	~~0.093~~	StDev =	n/a
0.248	RSD =	4.987 %	~~0.093~~	RSD =	n/a %

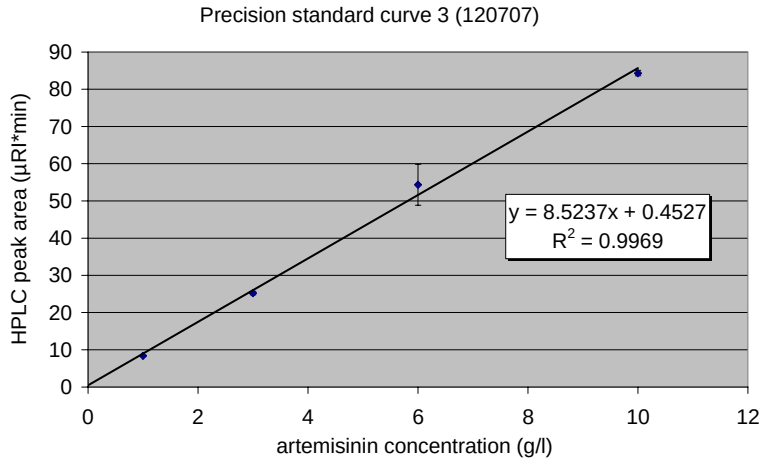
Analysis of data set 3

- Areas of the artemisinin and internal standard peaks were tabulated, and averages were obtained for the replicate injections:

Precision assay data set 3							
		peak areas (µRIU*min)					
		artemisinin			Internal standard		
		Set 1	Set 2	**average**	Set 1	Set 2	**average**
SC	1	8.358	8.365	**8.362**	7.861	9.134	**8.498**
	3	24.921	25.583	**25.252**	7.806	8.837	**8.322**
	6	50.443	58.216	**54.330**	7.734	10.099	**8.917**

	10	83.835	84.85	**84.343**	7.368	7.867	**7.618**
		injectn 1	injectn 2	average	injectn 1	injectn 2	average
A	1	67.904	67.741	**67.823**	7.155	7.171	**7.163**
	2	67.324	68.512	**67.918**	7.292	7.326	**7.309**
	3	77.256	77.54	**77.398**	7.714	7.983	**7.8485**
B	1	16.882	16.81	**16.846**	8.063	8.055	**8.059**
	2	17.605	17.699	**17.652**	8.695	8.649	**8.672**
	3	17.433	16.062	**16.748**	8.224	7.577	**7.9005**
C	1	4.364	4.201	**4.2825**	8.362	8.402	**8.382**
	2	4.3429	4.603	**4.473**	9.328	9.361	**9.3445**
	3	4.313	4.28	**4.2965**	8.462	8.251	**8.3565**
D	1	19.409	19.16	**19.285**	9.957	10.091	**10.024**
	2	18.334	18.083	**18.209**	9.108	9.298	**9.203**
	3	18.491	18.127	**18.309**	8.817	9.017	**8.917**
E	1	4.309	4.157	**4.233**	9.214	9.382	**9.298**
	2	5.055	4.897	**4.976**	10.67	10.79	**10.73**
	3	4.709	4.513	**4.611**	9.632	9.414	**9.523**
F	1	1.316	1.207	**1.2615**	8.084	8.119	**8.1015**
	2	1.356	1.295	**1.3255**	8.784	8.946	**8.865**
	3	1.187	1.268	**1.2275**	8.293	8.341	**8.317**

The standard curve were plotted (SC1, 3, 6 and 10), using average values from both standard curves. The standard deviations are displayed as error bars.



The equations from this line of best fit were used to calculate the amount of artemisinin in each sample.

Sample set 3

sample A (8mg/mL)

7.904	mean =	8.282
7.915	StDev =	0.645
9.027	RSD =	7.793 %

sample B (2mg/mL)

1.923	mean =	1.951

2.018 StDev = 0.058
1.912 RSD = 2.984 %

sample C (0.5mg/mL)
0.449 mean = 0.457
0.472 StDev = 0.012
0.451 RSD = 2.723 %

sample D (2-fold dilution of extract)
2.209 mean = 2.129
2.083 StDev = 0.070
2.095 RSD = 3.275 %

sample E (8-fold dilution of extract)
0.444 mean = 0.487
0.531 StDev = 0.044
0.488 RSD = 8.944 %

sample F (32-fold dilution of extract)
0.095 mean = 0.096
0.102 StDev = 0.006
0.091 RSD = 6.077 %

Analysis of all data sets

- Repeatability values were calculated by averaging the RSD values obtained for three data sets, for a given sample. These values are presented in the table below and it is clear that for a given sample, the calculated concentration varied by less that 6% over a day-long assay.
- The mean concentration values calculated after each day of assays (three assays per day, minus some failed injections) are presented in the table below (red value is not a mean, but the only one of the three samples that injected successfully). The first two values are generally closer to one another than the last one. This is believed to be due to the samples degrading with time. The first two sets were run on consecutive days, while the last set was run approximately 5 days later. This is more pronounced for the less concentrated samples.
- Intermediate precision values were obtained by calculating the RSD of the mean values for a given sample type. These are also presented in the table below. It is clear that the differences are high, particularly for the samples with lowest concentrations of artemisinin. This is almost certainly because the samples are degrading with time. This issue will need to be borne in mind when applying this assay in the field.

	mean 1	mean 2	mean 3	repeatability (RSD)	intermediate precision (RSD)
sample A	8.284	8.280	8.282	4.15 %	0.02 %
sample B	2.078	2.015	1.951	3.44 %	3.16 %
sample C	0.642	0.607	0.457	4.28 %	17.27 %
sample D	2.476	2.339	2.129	5.82 %	7.55 %
sample E	0.649	0.701	0.487	5.82 %	18.18 %
sample F	0.236	0.217	0.096	5.53 %	41.41 %

5.5 Detection & Quantitation Limits

art peak area

	A			B			C	av	sd
L1	7.517	7.405	7.353	9.512	9.581	9.426	8.817	8.515857	1.051177
L0.5	3.785	3.694	3.671	4.773	4.815	4.82	4.446	4.286286	0.548856
L0.25	1.895	2.057	1.84				2.248	2.01	0.183465
L0.125	0.921	0.908	0.902	1.156			1.504	1.0782	0.260809
L0.1		0.726	0.733	1.047			0.796	0.8255	0.150985
L0.05	0.356	0.452		0.545			0.605	0.4895	0.109009
L0.025	0.242	0.144		0.271			0.206	0.21575	0.054726
blank	0	0	0	0			0	0	0
4A	15.438	15.79	15.61				19.952	16.6975	2.174421
1A		3.953	3.928	4.177			4.95	4.252	0.478611
0.5A	2.023	2.04	1.678	2.768			2.563	2.2144	0.442371
.25A	1.001	1.138	1.077	1.425			1.174	1.163	0.160491
.125A		0.51					0.632	0.571	0.086267
.1A		0.48					0.56	0.52	0.056569
.05A	0.18	0.253	0.206	0.209			0.276	0.2248	0.038816
.025A	0.154	0.246	0.199	0.139			0.214	0.1904	0.043844
mobile phase				0.155				0.155	

art peak height

	A			B		C		
L1	29.895			37.05		34.599	33.848	3.636139
L0.5	15.227			19.03		17.534	17.26367	1.915858
L0.25	7.751					9.003	8.377	0.885298
L0.125	3.872			4.849		6.726	5.149	1.450458
L0.1	3.271			4.025		3.18	**3.492**	0.463829
L0.05	1.638			2.006		2.603	2.082333	0.487008
L0.025	0.832		0.607	1.086		0.753	**0.8195**	0.20063
blank	0.35	0.351	0.255			0.332	0.322	0.045512
4A	55.325					70.989	63.157	11.07612
1A	15.407			16.059		18.96	16.80867	1.891415
0.5A	7.765			10.44		9.591	9.265333	1.366913
.25A	3.836			5.196		4.873	**4.635**	0.710551
.125A	2.187	2.249				2.549	2.328333	0.193601
.1A	2.131	1.915				2.214	2.086667	0.154351
.05A	0.783	0.838	0.8	0.878		1.073	**0.8744**	0.116907
.025A	0.587	0.767	0.699	0.565		0.648	0.6532	0.082518
molile phase				0.362			0.362	

noise defined as 0.322
detection limit thus becomes .322x2.5 =0.805
quantitation limit becomes 0.322 x 10= 3.22

nab peak

	A			B		
L1	13.073	13.291	13.261	13.308	13.262	13.328
L0.5	13.705	13.844	13.802	13.881	13.781	13.742
L0.25	13.379	13.466	12.83	17.358		
L0.125	13.464	13.709	13.39	13.765		
L0.125	13.414	13.594	13.016	13.645		
L0.05	14.563	14.756	8.509	13.789		
L0.025	12.812	12.762		13.11		
blank	13.039	12.812	12.97	18.361		
4A	14.049	13.996	13.702	15.464		
1A		13.668	13.88	14.098		
0.5A	14.362	14.63	12.487	14.84		
.25A	13.902	13.834	13.573	14.145		
.125A	16.868	17.066		17.838		
.1A	16.709	16.997		18.646		
.05A	13.867	13.915	13.224	14.251		
.025A	14.652	14.812	11.645	13.808		

N.B. samples highlighted in yellow failed to inject properly and have been ignored.

Detection limit- peak height above 0.805 was seen in samples of concentration of 0.025mg/mL artemisinin added to a lemon balm extract (peak height is 0.82 +/- 0.2). Quantitation limit- peak height above 3.22 was seen in samples with 0.1mg/mL artemisinin added to lemon balm extract (peak height is 3.49 +/- 0.46).

For the RAE this equates to dilutions of extract of 1 in 80 for detection limit and 1 in 16 for the quantitation limit, as discussed in the preceding Section.

5.6 Robustness & System Suitability

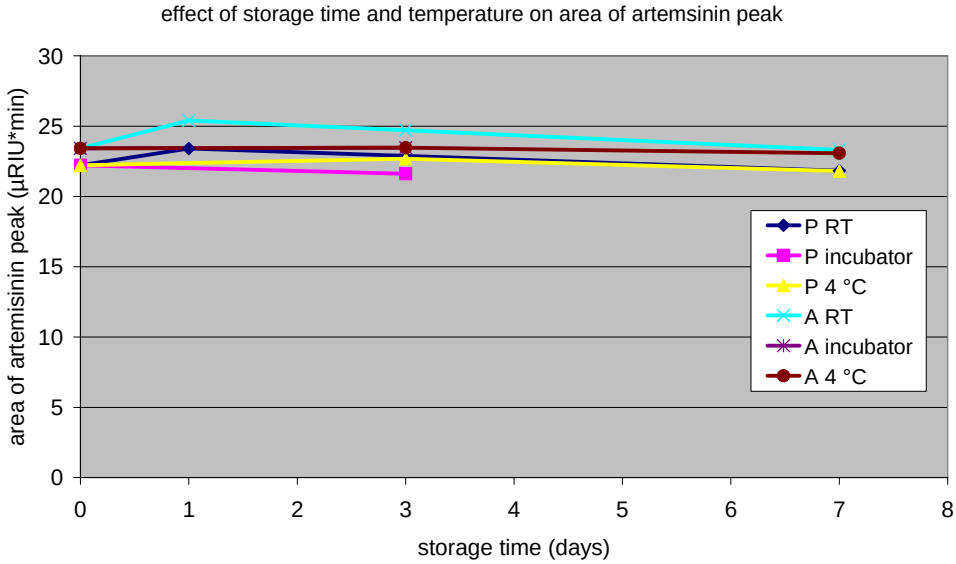
Varying mobile phase composition and flow rate

Conditions (% MeCN)	Artemisinin standard (RA) ID	Artemisinin peak RT (mins)	area (µRIU*min)	*Artemisia* plant extraction (RAE) ID	Artemisinin peak RT (mins)	area (µRIU*min)
60%, 0.5ml/min	2	19.26	8.794	3	19.138	20.999
65%, 0.5 ml/min	5	15.481	7.1	6	15.472	20.95
70%, 0.5ml/min	8	12.975	7.392	9	12.949	19.988
50%, 1 ml/min	11	16.74	3.294	12	16.621	9.528
55%, 1 ml/min	14	12.349	3.994	14	12.28	10.248
60%, 1 ml/min	17	9.577	3.594	18	9.579	10.564

- For the artemisinin standard (RA)
    - The signal : noise ratio appeared excellent with 60% acetonitrile, but slightly less good at 65, 70, 50 and 55%, although the peak was still clearly distinguishable. As expected, the retention time was decreased at higher concentrations of acetonitrile (with identical flow rate)
    - Increasing the flow rate lead to a significant reduction in retention time for the artemisinin peak.
    - The peak area (in µRIU*min) is halved when the flow rate is doubled. This is due to the way that the peak area is calculated, and means that it is important that the same flow rate is used for calibration standards and samples.
- For the *Artemisia* plant extract (RAE)
    - The effects of mobile phase on peak area and retention time seen for pure artemisinin (above) were also observed in the RAE samples.
    - Separation of the artemisinin peak from other peaks in the extract was good (close to baseline) for all of the samples. The least good resolution was seen at 50 and 70% acetonitrile. The best resolution was seen at 60% acetonitrile, at 1 and 0.5 ml/min.
    - Resolution of the artemisinin peak at 60% acetonitrile was equally good at 1 and 0.5 ml/min.

Stability of samples prior to HPLC-RI assay

- The stability of samples of RAE and RA was examined over 7 days of storage at 4 °C, room temperature or in an incubator at (?30 °C). Samples were prepared on 28/06/07 and run on days 0 and 1.
- The peak areas corresponding to artemisinin in the successful injections were plotted against storage time. In this graph (below) it is clear that the peak areas decrease by only a small amount over the week, at all storage temperatures tested.
- This means that samples are likely to be stable while waiting to be injected, even if they cannot be stored under temperature-controlled conditions



The assay is thus considered to be sufficiently robust for widespread use, provided that an internal standard is used each time to identify failed injections.

6. Conclusion

The HPLC-RI assay for artemisinin described above has thus been validated in accordance with the relevant ICH harmonized tripartite guidelines and was found acceptable under each criterion tested. Prior to application it is however recommended that validation of the assay be performed in one or more external laboratories, to ensure that inter-laboratory variations are not significant.