Project Title	**Developing an alternative UK industrial crop Artemisia annua, for the extraction of Artemisinin to treat multi-drug resistant malaria. (Council funding cross sector)**
Project number:	**CP 44**
Project leader:	Ian Flockhart [Botanical Developments Ltd]
Report:	First Annual report, April 2007 (received August 2007)
Previous report	None
Key staff:	Colin Hill - Commercial extraction Trevor Robinson – Crop agronomy, Frontier Nigel Dungey – Seed production, Humber VHB Steven Bentley – Parent breeding, NIAB Christopher Atkinson – Plant physiology, EMR Jack Woolley – Analytical analysis
Location of project:	NIAB, East Malling Research, De Montfort University, Frontier, Humber VHB.
Project coordinator:	David Hand, Humber VHB
Date project commenced:	1st April 2006
Date completion due:	1st April 2010
Key words:	*Artemisia annua*, seed production, agronomy, breeding, variety trials, yield, nutrient effects, fertiliser.

The results and conclusions in this report are based on a series of experiments conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, the biological nature of the work dictates that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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1 Grower Summary

Headline

In the first year of this project LK0822, considerable progress was made on the following research priorities:-

- Systematically evaluate the best material collected in 2005.
- Confirm its potential using improved analytical techniques.
- Characterise the flowering habit of the material for seed production.
- Make crosses using the best material for evaluation in 2007.

Improved material was not available in 2006 from the objectives above, therefore agronomic objectives were being addressed using imported germplasm.

Background and expected deliverables

Artemisia annua is being investigated here for its potential use in the treatment of malaria. This is achieved by the extraction of the active pharmaceutical ingredient (API) artemisinin from the leaves and young stems of the plant. The active ingredient cannot currently be economically synthesised using bulk fermentation or chemical synthesis. Extraction from plants remains the most economically feasible source of this medicine for the foreseeable future.

This 4 year LINK project follows on from a successful one-year, DEFRA funded project NF0613. The trial results indicated that certain *Artemisia annua* lines grew well under the UK weather conditions (summer 2005) and a few of the better adapted lines also had high levels of artemisinin. However, there is a need for significant work to better characterise this material for UK conditions and develop cost affective planting and harvesting techniques.

The success of major crops within the UK is dependent on an understanding of the many interacting variables affecting productivity. These may have been developed and refined over many years of crop development. Clearly, it will not be possible to perfect our understanding of this crop and its agronomy in a four year study but significant progress should be possible.

The work reported in NF0613 concluded that a fully integrated UK production chain was possible but it would be imperative to have a secured supply of fully characterised germplasm. The germplasm tested in 2005 indicated that this could be achieved and that it would be necessary to develop well controlled experiments and analytical techniques to characterise the germplasm.

Summary of the project and main conclusions

- Systematically evaluate the best material collected in 2005

Approximately 700 plant lines were screened with samples of the 1015 material run as controls (harvested and prepared at the same time). Most of the lines tested had artemisinin contents of less than 0.5%. The results for the line 1015 were very variable between 0.95 and 2.59% and seemed to be dependent on each analytical

run. Therefore it was necessary to make selections relative to the control used in each analytical run.

Project NF0613 had indicated the need to develop a better screening method for the artemisinin content of plant material. The development of screening technique, whilst also selecting germplasm was always going to be difficult. However, working closely with De Montfort University, it was possible to re-analyse the selected samples over the winter period using improvements made to the new rapid screening technique. This was replicated and produced a more consistent result between the samples tested. There was also a greater consistency between the samples from the different sampling dates.

- Confirm its potential using improved analytical techniques

For the purposes of this project, an extraction method was needed that was relatively simple (to reduce preparation time), clean, economical and artemisinoid-specific.
Applied Analysis, working with BDL, devised a method that employed the use of toluene as an extraction solvent – 500 mg of dried herb were macerated overnight in 25 ml toluene. A 2ml sample of this extract was then loaded onto a solid-phase-extraction (SPE) column and pure artemisinin was sequentially eluted with a gradient of heptanes and ethyl acetate.

This method works very well, but for high-throughput screening, it was found to be a little too time-consuming; the SPE clean-up was a major bottleneck. Hence considerable work was undertaken to develop a new solvent extraction method and develop the use of HPLC.

Using HPLC the analysis performed in summer 2006 was dogged by variation in replicated analyses. It was noticed that data were more variable a) towards the end of a long sequence (50+ runs on the HPLC), and b) when the Security Guard pre-column had not been changed for a number of runs. Appropriate control methods have now been implemented. The repeated parent analysis results show the progress that has been made and the potential artemisinin content of the selected lines.

- Characterise the flowering habit of the material for seed production

The plants were received by EMR as rooted cuttings from NIAB in August 2006. There were 27 clones with 3-7 individual replicate plants for 26 of the clones and 22 plants for clone 1 (1015).

After establishment, day length was shortened naturally by switching off the supplementary radiation on the 3rd of November (2006). Natural day length at this time was approximately 10 hr. Lighting was switched on for 4 hr a day between 9 am and 1 pm. Flowering started on 24th December (2007).

Using this method of evaluation showed considerable variation between and within the lines. It was felt that the characterisation of plant under glass during the summer as demonstrated in 2005 may provide better data to inform seed production.

- Make crosses using the best material for evaluation in 2007.

The approach used the plants originally in the experiment to determine time of flowering, once they had reached the first open flower stage. Crosses were carried out by placing both parents side by side and wrapping them in a layer of horticultural fleece (Figure 2). The plants were then placed into a further glasshouse chamber maintained at 20^0C (natural radiation and day length). The plants were left for 6-8 weeks to allow flowering to be completed and seed set. During this period plants were shaken twice weekly to disperse the pollen and aid fertilisation; watering during this phase was kept to a minimum.

The crossing programme has been a very effective demonstration of what can be achieved with this material and has provided the potential to start crop improvement for the UK. It was not possible to make all of the most desired crosses but some potentially very good combinations have been made.

Clearly, with the improved analytical techniques that have been developed, it will be possible to identify better parent material for making crosses in future years. In the future it will be possible to use fewer parents and replicate the parents several times in different growing conditions to achieve the ideal flowering stage for both parents at the same time. This will allow all the most desired crosses to be achieved with greater ease.

Financial benefits

Humber VHB used four lines from the 2005 project to evaluate the commercial potential for seed production. These lines have produced seed for field testing in 2007 and further seed production contracts are under discussion.

The UK produced seed has already proven to be of higher quality than that previously imported. Clearly in 2007/08 Humber VHB will be able to produce commercial seed based on the testing of the lines produced by EMR.

Frontier Agriculture has invested considerable effort into the establishment of a field crop using traditional vegetable transplanting techniques. This will be reported in 2008.

Action points for growers

There are already several commercial growers involved in this project, though the work is still at a relatively early stage of development. However the significant progress already made suggests that this opportunity will start to provide significant benefits to growers before the end of the project.

2 Science Section

2.1 Introduction

Artemisia annua is a potential new biopharmaceutical crop with no previous history of large scale or even small scale cultivation in the UK outside of botanic and private gardens. It is being investigated here for its potential use in the treatment of malaria. This is achieved by the extraction of the active pharmaceutical ingredient (API) artemisinin from the leaves and young stems of the plant. This is converted into more water soluble derivatives and formulated into oral medication. The active ingredient cannot currently be economically synthesised using bulk fermentation or chemical synthesis. Extraction from plants remains the most economically feasible source of this medicine for the foreseeable future. The need for this medicine is urgent due to the collapse of most currently used medications through development of multiple drug resistance by the malaria parasite.

This 4 year LINK project follows on from a successful one-year, DEFRA funded project NF0613. Significant work has been carried out elsewhere and has been reported in the literature during the last 30 years, but much of this work reported conflicting results with respect to many aspects of cultivation, particularly with respect to plant physiology and API production. A literature search concluded that many of the conflicting results were due to the range of germplasm used and the diverse regions of the world where testing had been conducted. The trial results indicated that certain *Artemisia annua* lines grew well under the UK weather conditions (summer 2005) and a few of the better adapted lines also had high levels of artemisinin. However, there is a need for significant work to better characterise this material for UK conditions and develop cost affective planting and harvesting techniques.

The success of major crops within the UK is dependent on an understanding of the many interacting variables affecting productivity. These may have been developed and refined over many years of crop development. Clearly, it will not be possible to perfect our understanding of this crop and its agronomy in a four year study but considerable progress should be possible.

The work reported in NF0613 concluded that a fully integrated UK production chain was possible but it would be imperative to have a secured supply of fully characterised germplasm. The germplasm tested in 2005 indicated that this could be achieved and that it would be necessary to develop well controlled experiments and analytical techniques to characterise the germplasm. The key objectives for 2007 were:-

- Systematically evaluate the best material collected in 2005
- Confirm its potential using improved analytical techniques
- Characterise the flowering habit of the material for seed production
- Make crosses using the best material for evaluation in 2007.

Improved material is not yet available from the objectives above, therefore agronomic objectives are being addressed using imported germplasm; this can be summarised as:

- Development of a robust agronomic guide for growers.
- Improved harvest protocols

3 Materials and Methods

3.1 Work Package 1, Developing a rapid artemisinin assay – Led by De Montfort University

Introduction

A large number of samples were to be generated on a regular basis by the work described in WP 2. It was therefore necessary to develop a rapid, reliable, and economical procedure that would bring us from dried herb, to useful data. Discussions with consortium members raised a number of potential methods (densitometric thin layer chromatography [TLC], high performance liquid chromatography-diode array detection [HPLC-DAD], HPLC-mass spectrometry [HPLC-MS], immunological techniques), many of which had already been successfully trialled in previous work by consortium members. However, in the interests of simplicity (in order to reduce preparation time) for this large-scale analysis of samples, a modified extraction technique was developed to prepare samples for analysis by HPLC-DAD and HPLC-MS. In addition, a new TLC method was developed, and a new rapid gradient method is being trialled for use on HPLC-MS.

Procedure

When samples are received from NIAB, they are immediately logged onto a shared database for tracking purposes. Then, the samples are – as a batch – powdered in a pestle and mortar, and macerated as a 1 in 10 extract in acetonitrile for 24 hr in a cool dark room, with gentle rotation (40 rpm).

Following this, the liquid extract is removed and placed into a capped clear glass autosampler vial. Our SOP states that the sample must be either analysed immediately, or otherwise stored in a refrigerator, protected from light, and analysed within 48 hr at the latest. Before analysis, samples are removed from the refrigerator and allowed to stand for 1 hr at room temperature.

Instrumental

For the quantification of artemisinin, samples are analysed by reverse-phase high performance liquid chromatography coupled to a diode-array-detector (HPLC-DAD). An isocratic (single solvent) method is currently used, where the mobile phase consists of 60% HPLC-grade acetonitrile and 40% HPLC-grade H_2O. This is vacuum filtered through a nylon membrane (with a pore size of 0.45 μm) to remove particulate impurities, and then placed in a sonicator for 10 minutes to remove dissolved gases. The stationary phase is a Phenomenex Luna C18 250 x 4.6 mm, 5 µm, 100 Å column with a Security Guard pre-column (C18, 4 x 3.0 mm), also from Phenomenex. The pre-column is replaced every 50 runs. Flow rate is 1 ml/minute, and the oven temperature is set to 30°C. Although the DAD records all ultraviolet and visible wavelengths, artemisinin is quantified at 214 nm.

Quality Control

For quantification purposes, the response factor of artemisinin was calculated (see report at the end of this document) and a calibration curve of artemisinin in this system across a range of concentrations is also generated.

One aspect found to be vital, was the importance of keeping track of the column's performance by calculating theoretical plates (*N*). *N* is a numerical measure of how good a column is at resolving closely eluting peaks (i.e. single compounds) as calculated by a simple formula: This will be discussed in more detail later

N = ([retention time of compound/peak width]^2) x 16

As described above, variation in reported artemisinin levels, even across replicates and in control, dogged much of the work. It seems that this could be traced to deterioration in *N*: when plates dropped below 30,000, results were very variable (see attachment). A new column would have an *N* (calculated for artemisinin) of 50000 – 60000. De Montford now include, with certificates of analysis, a report of column performance calculated before and after each batch analysis.

Other Techniques

Besides HPLC, a new TLC method has been developed which separates artemisinin better from volatile components (artemisinin, being a sesquiterpene, is difficult to separate by TLC from the monoterpenes which give the plant its characteristic odour). However, this is only used as a starting point for analysis, because it is only semi-quantitative.

At De Montfort, there is an HPLC-MS in-house, which can perform both ESI (electrospray ionisation) and APCI (atmospheric pressure chemical ionisation). this has been used in its ESI form to qualitatively analyse both pure and semi-pure artemisinin, as well as extracts of dry and fresh *A. annua*. Artemisinin can now be identified by its fragmentation pattern, and AECS have provided a number of other reference compounds that will be analysed by MS and NMR spectroscopy, so that very soon, quantification of other precursor/degradation products typical of *A. annua* extracts will be possible (see below), which will allow much more information about the new cultivars being generated to be obtained.

Further work on improvement of analytical characterisation

De Montfort are still working on improving HPLC analysis time in terms of solvent usage and run time. Currently, a method has been developed by them for the HPLC-MS. The new method uses a two-solvent gradient system with a narrow-bore column (150 x 2.1 mm, 5 µ). Narrow-bore columns, so called because their internal diameters are narrower than standard columns, have the advantages of being generally highly sensitive, requiring much less solvent (typically 0.2 ml/minute, rather than 1 ml/minute) and in this case, the new method also allows the run time to be shortened by 8 minutes. It seems that this method separates most compounds well, particularly flavonoids, but still needs some modification. However, it is anticipated that this could become the standard analytical method, and the reduced flow rate would mean that standard analyses, even of large scale batches, could be rapidly and economically analysed by HPLC-MS, which gives more information, and is more selective and sensitive, than DAD.

In addition to artemisinin content, the content of certain precursor and breakdown compounds will be analysed. Since current theory suggests that the latter stages of artemisinin biosynthesis are not under enzymatic control, but rather are functions of environmental conditions, De Montford propose that considering the whole metabolic "photograph" that a chromatogram gives would tell both something of the genetic potential of a plant, coupled with information about the conditions in which it was grown.

Additional projects that are in development:

- An in-field semi-quantitative method, perhaps using Fast Red in a colorimetric test kit

- Initiation of shoot cultures – these would be very useful for quick nutritional analysis. We have produced sterile tissue cultures by placing sterile plant tissue onto Murashige and Skoog medium, supplemented with indole acetic acid (IAA) and kinetin (6-Furfurylaminopurine).
- By similar methods root cultures have now also been established

3.2 Work Package 2, Development of improved germplasm – Led by NIAB

NF0613 indicated that three of the 26 lines tested in 2005 had both high artemisinin content and good agronomic characteristics. Using the NIAB logging system for germplasm, these lines were identified as:- 1001, 1012, 1015, and 1019

Additional seed was collected from 5 other sources and was also tested for the first time; these were logged as 1049, 1048, 1026, 1046, and 1047.
Line 1015 came from a single plant source in 2005. This proved to contain the highest percentage of artemisinin in 2006. It was therefore propagated from cuttings and was used as the main control sample in all the analytical work.

Seed was sown in the glasshouse on 13/4/06. The seedlings were pricked out into trays at the two true leaf stage on the 28/4/06. These were then grown in a cool glasshouse to produce small plants which were transplanted into the field 17/05/06.

Artemisia annua seed is produced from out crossing plants. Therefore the progeny may be similar but they are all genetically unique. The plants were all planted as spaced plants. A target plant population of 200 plants was grown for each line. The large number of plants meant that initially only one sample per plant could be tested. The objective was to discard the poor material rapidly allowing replicated testing for only the material which appeared to have the highest potential.

Figure 1. Spaced plants; 17th June 2006

The first samples were harvested in late July. Generally, line 1015 produced the best results at each analysis, but this showed significant variation between the samples tested. After analysis, only those lines with no lower than 10% of the artemisinin content of line 1015 were selected. This was done on a run by run basis. The selected material was re-sampled a further two times during the season (late August and September) to provide confirmation of the result.

For the work to be initiated at EMR it was necessary to provide them with rooted cuttings by late August. It was therefore necessary to take cuttings from the best plants after the initial analysis. This made a total of 32 lines. These were reduced to 27 lines after further analysis. For security of the selected material, propagated plants were maintained at both NIAB and EMR to prevent loss of a key parent lines.

Project NF0613 indicated that there could be significant differences between the samples tested from the same plant. In an attempt to reduce the sample variation a standard operating procedure was developed. This was to detail the way in which the sample was prepared and analysed. This document has been updated for the 2007 harvest to develop best practice. Considerable effort was also made to improve the analytical methodology (see below).

3.3 Work package 3 Agronomy and seed production led by EMR and Humber VHB and Frontier

Crossing programmes and an investigation of flower initiation have been led by EMR. EMR have been particularly concerned with crossing programmes and investigation of flower initiation. The work plan at EMR for 2006/07 was extended beyond providing relevant plant flowering data to provide the consortium with new, potentially improved *Artemisia* germplasm. To achieve this aim, EMR carried out crosses as directed by NIAB, using the parent lines available in 2006 (Table 1). The development of the breeding strategy protocol was developed by NIAB.

The approach used the plants originally used in experiments to determine time of flowering, once they had reached the first open flower stage. They were then removed from the flower induction environment (environmentally controlled glasshouse chamber) and placed into a separate glasshouse compartment kept at ambient temperature. Decisions were made, as appropriate, to attempt to reduce the speed with which different clones developed into the floral state, so that key relevant clones were synchronised for crossings, as outlined in the crossing protocol.

Crosses were prioritised from 1 to 5 (Table 1), with 1 being of high priority and 5 being of lower priority. Crosses were carried out by placing both parents side by side and wrapping them in a layer of horticultural fleece (Figure 2). The plants were then placed into a further glasshouse chamber maintained at 20^0C (natural radiation and day length). The plants were left for 6-8 weeks to allow flowering to be completed and seed set. During this period plants were shaken twice weekly to disperse the pollen and aid fertilisation; watering during this phase was kept to a minimum.

Once the plants had begun to turn brown, all the flowers (plus some leaf material) from both parents were collected and bulked together, and dried in an oven for 48 hr at 24^0C. The bulk material was then passed through a 2 mm sieve to separate the seed from as much of the leaf material as possible. Collected seed was stored in brown paper envelopes and sealed within bags with a desiccant and kept in a cool cabinet at 4^0C until dispatch to NIAB.

Table 1. *Artemisia annua* crossing strategy priorities and the number of crosses achieved at East Malling Research within each crossing priority group

Crossing Priority*	Parent 1	Parent 2	Clones within each grouping	Number of crosses
1*	1015 (line 1)	All groups	All lines within each group	15
2	Group B (minus 1015)	Group A	A: 2, 46, 81, 85, 505, 604, 682 B: 301, 302, 303, 304 305, 322	12
3	Group B (minus 1015)	Group C	B: 301, 302, 303, 304 305, 322 C: 508, 521, 535, 578, 579	2
4	Group A	Group C	A: 2, 46, 81, 85, 505, 604, 682 C: 508, 521, 535, 578, 579	11
5	Group B (minus 1015)	Group D and Group E	B: 301, 302, 303, 304 305, 322 D: 352, 344, 394, 446 E: 324	1

Note *Crosses are prioritised with 1 being the highest and 5 the lowest.

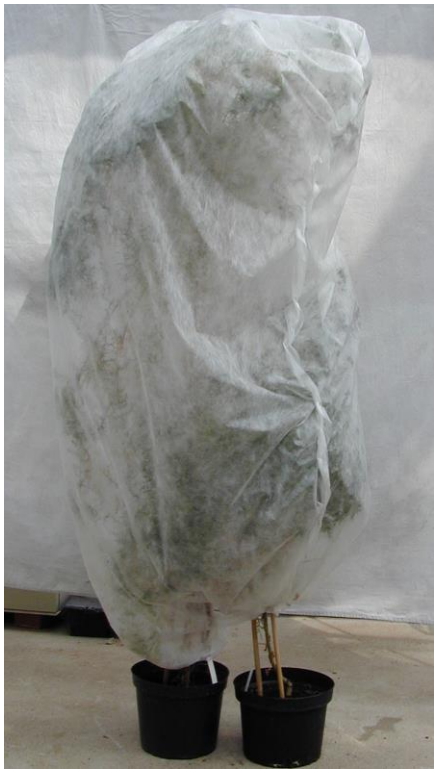

Figure 2 - Two different clones wrapped in horticultural fleece for crossing

3.3.1 Determining the flowering times of geographically diverse *Artemisia annua* clones, led by EMR

The plants were received as rooted cuttings from NIAB in August 2006, and were potted up into 10 l pots and placed on benches pot thick in an 8 m x 3.2 m glasshouse compartment at 20^0C under natural radiation levels but day length was maintained above 15 hr through the use of supplementary extension lighting. There were 27 clones with 3-7 individual replicate plants for 26 of the clones and 22 plants for clone 1 (1015).

Subsequently, day length was shortened naturally by switching off the supplementary radiation on the 3rd of November (2006). Natural day length at this time was approximately 10 hr. The end wall of the glasshouse compartment ('short-day') and the door access had already been blacked out through the use of thick black polythene and the side screens of the compartment were also shut during the hours of darkness, to minimise any impact of light pollution. The location of the compartment was also selected to avoid and unwanted light pollution during the shortening photoperiod. Supplementary lighting was switched on for 4h a day between 9 am and 1 pm.

Half of the plants of clone 1 (1015) were moved to a 16 hr day length compartment to delay flowering and so enabling them to be crossed with other clones flowering later; these were returned back to the natural day length conditions on 21st November (2006). Clone 258 was placed into 16 hr day length conditions on 3rd November as they had started to flower.

Flowering started on 24th December (2006) (Clone 2). Flower assessment records were taken of each individual of each clone twice weekly. Flowering records include the position of flowers i.e. whether flowers were found at the top, middle or base of the plant. The stage of flowering was also recorded, 3 stages were used, when flower spikes started to develop (1), when individual flowers could be easily recognised (2), when the first flower was open (3). When the plants reached the 'first flower open' stage, they were removed from the short-day compartment ready to be used for the crossings program. The flowering experiment was terminated at the end of February 2007 when the majority of all the selected clones had flowered.

3.3.2 Seed production – Led by Humber VHB

Using the best material identified in project NF0613, Humber VHB attempted to evaluate the seed production potential of several lines. The material used was from the populations 1015 and 1001. For each line, 160 plants were propagated from the original mother plants. These were grown under glass with supplementary lighting until the plants were 12" tall. They were then put into a 12 hr day length to initiate flowering.

For good pollination the plants were kept in close proximity and shaken every day to facilitate pollen spread.

3.3.3 Agronomy – Led by Frontier

NFC0613 indicated that field production in the UK would be possible but that considerable work was required to make commercial field production cost effective. Two critical issues were the establishment of the crop and weed control.

Establishment of the crop from seed would be the most cost effective strategy. Large quantities of seed were only available from imported stocks of variable quality. However this provided material for experimental work until quality UK produced seed can be made available. Project NFC0613 indicated that rapid plant growth had only occurred from mid May in 2005 and this had corresponded with the recorded increased soil temperatures. Seed was therefore sown at NIAB and four locations on commercial farms.

The seed was drilled very shallow due to the small seed size but covered so that a range of pre and post-emergent herbicides could be evaluated.

3.4 Work Package 4. Harvesting, product stabilisation and secondary products; led by Frontier

Development of a more efficient delivery system is being addressed. In the current project, the crop has been harvested in several different ways and then dried ready for processing. This needs to be developed for large scale commercial farm production systems and therefore, the following issues will be evaluated:- This Work Package is at an early stage of development and will be addressed in the next years of the project.

4 Results and Discussion

4.1 Artemisinin content of material selected by NIAB

Approximately 700 plant lines were screened with samples of the 1015 material run as controls (harvested and prepared at the same time). Most of the lines tested had artemisinin contents of less than 0.5%. The results for the line 1015 were very variable between 0.95 and 2.59% and seemed to be dependent on each analytical run. Therefore it was necessary to make selections relative to the control used in each analytical run. The lines selected for repeat analysis are shown in Table 2. The lines (highlighted in yellow) were those finally used by EMR for crossing.

The results indicated that there was a very large variation between the samples analysed, making the final selection very difficult. However, to continue with the other planned work it was necessary to make a selection based on the best data available at the time. The material selected for testing and crossing at EMR was therefore rather more variable in potential artemisinin content than had been initially planned.

Project NF0613 (or NFC0613) had indicated the need to develop a better screening method for the artemisinin content of plant material. The development of screening technique, whilst also selecting germplasm was always going to be difficult. However, working closely with De Montfort University, it was possible to re-analyse the selected samples over the winter period using improvements made to the new rapid screening technique. This was replicated and produced a more consistent result between the samples tested. There was also a greater consistency between the samples from the different sampling dates.

Table 2. Parent analysis, % artemisinin

Candidate parent plants

Parent plants

Three sampling dates during the season

	First analysis				Average of two samples Second analysis			
Sample No	**1st Harvest**	**2nd Harvest**	**3rd Harvest**	**Average**	**1st Harvest**	**2nd Harvest**	**3rd Harvest**	**Average**
1	2.56	1.55	2.18	**2.10**	1.95	1.95		**1.95**
2	2.00	1.54		**1.77**	1.66			**1.66**
46	1.18	2.00		**1.59**	1.74	2.16		**1.95**
78	0.85	0.94	1.45	**1.08**	0.86	0.82		**0.84**
81	0.87	1.50	1.65	**1.34**	1.26	1.34	1.31	**1.30**
85	0.85	1.10	1.11	**1.02**		1.36	1.03	**1.20**
258	1.07	0.37	1.04	**0.83**	0.55	0.48	0.61	**0.54**
301	3.07	1.47	1.67	**2.07**	1.40	1.02	1.32	**1.24**
302	2.61	1.44	1.61	**1.89**	1.00	0.97	1.25	**1.07**
303	2.48	1.61	1.17	**1.75**	0.96	1.04	1.35	**1.12**
304	2.35	1.61	1.65	**1.87**	1.31	1.23	1.72	**1.42**
305	3.13	2.67	2.54	**2.78**	1.74	1.54	1.75	**1.76**
318	1.68	1.34	0.92	**1.31**	0.90	1.14	1.65	**1.23**
322	1.63	1.78	1.22	**1.54**	1.52		1.63	**1.57**
324	1.34	1.11	1.54	**1.33**	0.58	1.02	1.25	**0.95**
329	1.61	1.26	2.20	**1.69**	1.75	1.42	2.30	**1.82**
341	1.68	1.29	2.38	**1.78**	1.39	1.27	1.30	**1.32**
344	1.80	1.40	1.03	**1.41**	0.73	1.24	1.36	**1.11**

352	2.00	1.99	2.65	**2.21**	1.14	1.46		**1.30**
394	1.41	0.98	1.49	**1.29**	1.41	1.16	1.75	**1.44**
446	1.63	0.80	1.37	**1.27**	1.23	1.13	1.30	**1.22**
505	1.61	1.00	1.90	**1.50**	1.58	1.31	2.00	**1.55**
508	0.72	0.72	0.64	**0.69**	0.87	1.17	0.82	**0.95**
521	2.13	0.63	1.47	**1.41**	1.03	1.01	0.87	**0.97**
535	2.12	1.11	1.04	**1.42**	1.46	0.98	1.74	**1.39**
568	1.13	0.74	1.53	**1.13**	0.55	1.43	1.00	**0.99**
578	1.12	0.59	0.97	**0.89**	0.86	0.84	0.61	**0.77**
579	1.24	1.29	0.86	**1.13**	0.67	0.94	0.67	**0.76**
583	1.38	1.09	1.80	**1.42**		0.95	1.42	**1.18**
604	1.13	0.97	1.17	**1.09**		1.26		**1.26**
625	1.26	0.87	1.51	**1.21**		1.27	1.16	**1.21**
682	1.40	1.28	2.06	**1.58**		1.35	1.14	**1.24**

4.2 Improvements for Analysis of Samples

For the purposes of this project, an extraction method was needed that was relatively simple (to reduce preparation time), clean, economical and artemisinoid-specific.
Applied Analysis, working with BDL, devised a method that employed the use of toluene as an extraction solvent – 500 mg of dried herb were macerated overnight in 25 ml toluene. A 2 ml sample of this extract was then loaded onto a solid-phase-extraction (SPE) column and pure artemisinin was sequentially eluted with a gradient of heptanes and ethyl acetate.

This method works very well, but for high-throughput screening, it was found to be a little too time-consuming; the SPE clean-up was a major bottleneck. Toluene as a solvent was also considered not ideal for our purposes, although it does produce a very clean extract, this extract could not be used without SPE because, firstly, toluene is not very volatile and takes a long time to dry, and secondly, it was found that toluene itself has a large low-UV absorbance, and a retention time (under the artemisinin HPLC method) exactly that of artemisinin. The degree of absorbance is such that even a trace of toluene, not visible to the eye, obscured any artemisinin peak and led to false quantification of artemisinin.

A consideration of the solvents used by other research groups for the extraction of artemisinin found that the most widely used were toluene (Vandenberghe, Vergauwe et al. 1995); *n*-hexane (Bilia, Melillo de Malgalhaes et al. 2006), (Kumar, Gupta et al. 2004); *n*-hexane and acetonitrile (ElSohly and Croom 1990); chloroform (Van Nieuwerburgh, Vande Casteele et al. 2006); or light petroleum . A few different solvents were tested by simple maceration. A “compromise” solvent was reached, for use in high-throughput screening: acetonitrile. Over a 24-hr maceration, acetonitrile was not quite as efficient as a 1:1 mixture of methanol and dichloromethane (DCM), but was as efficient as ethyl acetate and more efficient than hexane. It was cleaner than methanol: DCM and much faster, as the extract, being HPLC-compatible, unlike DCM extracts, could simply be HPLC-ed without further ado. This method is similar to that of ElSohly *et al* (1990), who extract with hexane and then partition into acetonitrile to obtain clean artemisinin.

A range of extraction techniques have been employed; a survey of recent literature illustrates that, apart from simple maceration, the extraction process may be assisted with the use of an ultrasonic bath (Vandenberghe found that a 30-min sonication was equivalent in extraction efficient to a 24 hr maceration, when toluene was used). For the purposes of this study, a simple 24 hr maceration (for high-throughput screening) or a 60 min sonication (for day to day work) was considered most suitable. It may seem illogical that for high-throughput screening to use a 24 hr maceration, but it was found that this method needed less user preparation time than the 60 min sonication, as the sonicator tends to overheat and must constantly be refreshed with iced water to prevent possible solvent evaporation and sample degradation. A 24 hr maceration is simpler to prepare.

As has been explained earlier in this document, the analysis performed in summer 2006 was dogged by variation in replicated analyses. It was noticed that these data were more variable a) towards the end of a long sequence (50+ runs on the HPLC), and b) when the Security Guard pre-column had not been changed for a number of runs. A number of samples were initially re-tested, in triplicate, over a three-week period, as shown in the table below. Samples beginning with F were Frontier samples, those beginning with N were NIAB samples, and GBR was a sample of *A. annua* obtained from a local herbalist, and included for comparative purposes.

Table 3. Replicate analysis: testing for variation between repeat sample analyses

Sample	Week 1	Week 2	Week 3
	Artemisinin % w/w (SD)	**Artemisinin % w/w (SD)**	**Artemisinin % w/w (SD)**
FA	0.8 *(0.03)*	0.72 *(0.01)*	0.77 *(0.06)*
FB	2.1 *(0.08)*	1.98 *(0.11)*	2.06 *(0.03)*
FC	1.74 *(0.1)*	1.63 *(0.12)*	1.52 *(0.07)*
FD	0.75 *(0.02)*	0.75 *(0.02)*	0.81 *(0.07)*
GBR	0.2 *(0.004)*	0	0
N65	1.00 *(0.03)*	0.89 *(0.02)*	0.91 *(0.03)*
N200	1.65 *(0.1)*	2.27 *(0.6)*	1.9 *(0.25)*
N409	1.14 *(0.06)*	1.57 *(0.13)*	1.06 *(0.05)*
N812	1.54 *(0.07)*	1.93 *(0.44)*	1.50 *(0.07)*
N950	1.70 *(0.08)*	2.35 *(0.26)*	1.86 *(0.28)*
***N* at start of analysis**	**30027**	**22779**	**43233**
***N* at end of analysis**	**29198**	**20006**	**41453**
***N* loss**	**829**	**2773**	**1780**

It can be seen from the table that when column plates fall below around 30,000, variation in results is seen. As a result of this, a minimum acceptable plate count has been added to the method developed here: The aim is to maintain plates at least 40,000; if they drop below 35,000 the software will flag up these results as unreliable. the pre-column is changed every 50 runs.

Sample size was identified as a factor influencing variability, and powdered *vs*. non-powdered samples. It was noted that sample size (between 100 mg and 1400 mg) did not affect variability, as long as lower samples sizes were ground. Slight variability was observed when 100 mg samples were not ground, but no variability was seen when 100 mg samples were ground.

The use of HPLC-DAD for the quantification of artemisinin is by no means ideal: artemisinin has no strong chromophore, and therefore weakly absorbs UV at 214 nm. This absorbance is uncomfortably close to the absorbance of acetonitrile (and indeed all other reverse-phase HPLC solvents), a major component of the mobile phase. It is this low absorbance that has so far prevented us from improving resolution and shortening analysis time with a solvent gradient: as acetonitrile levels increase throughout a test gradient, the baseline drifts so far to high absorption as to render the quantification of artemisinin unreliable. Subtracting a blank run improves the chromatogram, but not sufficiently. As discussed earlier, the De Montfort team are in

the process of developing a quantitative gradient method suitable for use on a narrow-bore column on the HPLC-MS. Artemisinin is easily detected by ESI (electrospray ionization) MS, and this method is used routinely perform qualitative LC-MS of artemisinin samples and extracts of *A. annua*.

4.3 Flower initiation

There was a wide variation in the time at which the various clones reach the first flower stage (1) after the imposition of short-day (Figure 3), with clones 305, 303 and 535 not coming into flowering at all during the 120 days of the experiment. Clone 258 was the first to flower and flowered within 40 days of the imposition of the short day treatment; this clone had a prostrate growth habit and is notably different from the other clones. Clones 2, 46, 682, 304, 322, 508, 344, and 446 generally flowered 40-80 days after imposition of the short day treatment and 81, 85, 505 604, 301, 302, 521, 578, 579, 352, 394, and 324 generally coming into flower 80+ days after the start of the treatment. Clone 1015 came into flower 70-90 days into the experiment and those placed into short days 18 days later flowered between 95-120 days.

There were also differences in the degree of synchronisation between individuals within a clone, for some clones all individuals of that clone come into flower over a short time period, with as little as 3-10 days separating the first plant coming into flower and the last replicate plant of that clone coming into flower, (e.g. 258, 304, 505, 521, 352) whilst for others the time to first flowering was over an extended period with 40+ days separating the flowering time of individuals within that clone (e.g. clones 85, 322, 301, 578, 579, 344).

The position of the flowers on the plant also differed between clones (Table 4), which is another important factor for developing an effective crossing programme, as we believe it is more difficult to cross plants where one parent is flowering at the top and the other is flowering at the base. The clones tested in this experiment tended to have one of two flowering habits, there were those that generally flowered throughout the plant, i.e. clones 2, 46, 81, 258, 304, 324, 344, 394, 446, 505, 508, 579, 583, 1015; and those that flowered at the base first with vegetative growth further up or much later flowering, i.e. 85, 301, 302, 303, 322, 352, 521, 535, 578, 604, 682.

Figure 3. Number of days after imposition of short day lengths that the first flower of individual plants within each clone open. The bars represent the time period over which plants within the clone come into flower. Red bars represent clones where all individual plants within that clone have 'first open flower', green bars represent clones where not all individuals within the clone had open flowers by the end of the experiment.

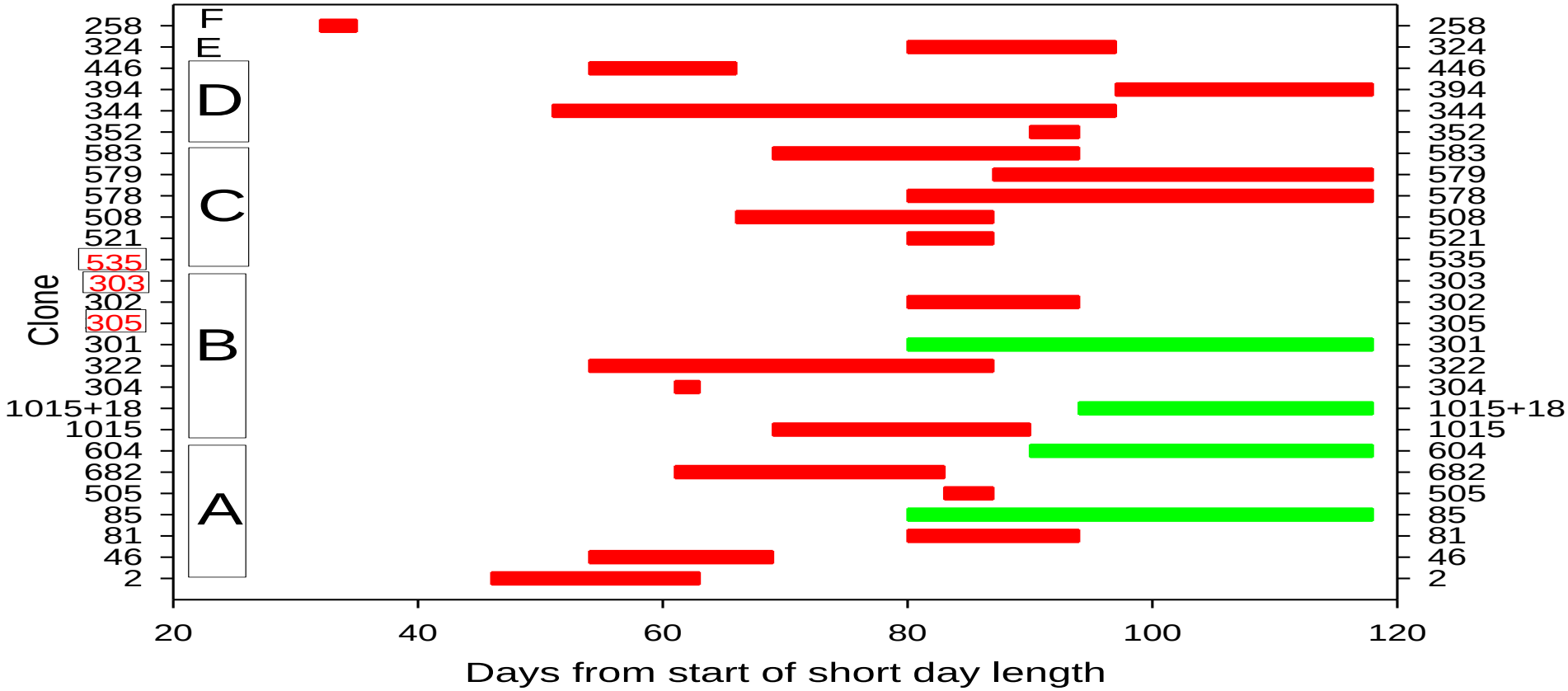

Table 4. Position that distinct flowers were found on the plants when the clones were at the first flower open stage

Clone	Position of flowers
2	Flowers distributed throughout the plant
46	Flowers distributed throughout the top 2/3 of the plant mainly – more advanced at the top.
81	Flowers distributed throughout the plant, with them being more advanced on the lower branches but not that further behind on the upper parts.
85	Flowers at bottom 1/3 of the plant only. Vegetative growth further up.
258	Prostrate growth habit – flowers throughout the plant and all at a similar stage in development
301	Flowers occurring on the bottom 2/3 of the plant. Those lower down are more advanced. Vegetative growth at the top only.
302	Flowers occur on lower 1/3 of plant with flowers at various stages of growth. Vegetative growth on upper 2/3 of the plant.
303	Flowers on the lower half of the plant. Even here there are very few flowers.
304	Flowers distributed throughout the plant, flowering more advanced at the top of the plant
305	/
322	Flowers generally on lower 2/3 of plant. Vegetative at the top
324	Flowers distributed throughout the plant. Majority on the top 1/2
344	Flowering distributed throughout the plant
352	Flowering on the lower 1/2 of the plant
394	Flowering distributed throughout the plant, flowering more advanced at the bottom of the plant
446	Flowering distributed throughout the plant, flowering more advanced at the top
505	Flowers distributed throughout the plant, the flowers are more advanced at the bottom of the plant
508	Flowers on the top 2/3 of plant
521	Flowers found on the bottom 1/3 of the plant. There are very few flowers. The plant is vegetative on the top 2/3
535	Very little flowering, what there is, is on the lower 1/3 of the plants
578	Flowers are occurring on the bottom 1/3 of the plant. Even here there are very few flowers. Vegetative growth on the top 2/3.
579	Flowers distributed throughout the plant. Flowers more advanced at the base of the plant
583	Flowers on the top 2/3 of the plant – the flowers are more advanced at the top of the plant.
604	Flowers on the lower half of the plant. Vegetative at the top
682	Flowers on the bottom 1/3 of the plant, flowering is patchy even here. Vegetative growth on the top of the plant, but it seems that the plant is turning reproductive slowly from the bottom of the plant
1015	Flowers distributed throughout the plant, although the majority are found at the top

The results showed that crossing parent material requires a good understanding of the material and during the winter the use of high quality glasshouse space. It may well be easier to manage the plants and be more cost effective to attempt all the crossing of material during the summer period. At NIAB glasshouse space equipped for Chrysanthemum growing, which can provide 100% blackout even during the summer months, was used in the research project NFC0613. In that project the hot summer conditions the differences between the lines tested was much less pronounced and flower initiation was very uniform on the whole plant.

Table 5. *Artemisia annua* crossings at East Malling Research during January 2007

Parent 1	Parent 2	Crossing Lines	Priority
1015	2	1015 x A	1
1015	446	1015 x D	1
1015	583	1015 x C	1
1015	508	1015 x C	1
1015	81	1015 x A	1
1015	505	1015 x A	1
1015	521	1015 x C	1
1015	578	1015 x C	1
1015	46	1015 x A	1
1015	324	1015 x E	1
1015	352	1015 x D	1
1015	394	1015 x D	1
1015	604	1015 x A	1
1015	258	1015 x F	1
1015	579	1015 x C	1
1015		Self	
2	304	A x B	2
2	322	A x B	2
682	322	A x B	2
46	322	A x B	2
46	304	A x B	2
682	302	A x B	2
682	301	A x B	2
505	322	A x B	2
85	322	A x B	2
81	302	A x B	2
85	322	A x B	2
85	303	A x B	2
322	521	B x C	3
301	521	B x C	3
2	508	A x C	4
46	583	A x C	4
46	508	A x C	4
46	521	A x C	4
505	508	A x C	4
81	583	A x C	4
81	521	A x C	4
682	521	A x C	4
85	578	A x C	4
46	578	A x C	4
81	508	A x C	4
302	394	B x D	5

The crossing programme has been a very affective demonstration of what can be achieved with this material and has provided us with the potential to start crop improvement for the UK. It was not possible to make all of the most desired crosses but some potentially very good combinations have been made, Table 5.

Clearly, with the improved analytical technique that have been developed, it will be possible to identify better parent material for making crosses in future years. In the future it will be possible to use fewer parents and replicate the parents several times in different growing conditions to achieve the ideal flowering stage for both parents at the same time. This will allow all the most desired crosses to be achieved with greater ease.

The weight of seed harvested has facilitated a start in testing the crosses using fully replicated trials in 2007. These can be both sampled for analysis and harvested to determine the total leaf yield. At the same time, all the parents used for the crosses are being retested. These have been propagated using cuttings and also form part of a replicated trial. Analysis from both the parents and the crosses will allow us to understand the level of heritability for artemisinin.

4.4 Humber VHB commercial seed yields.

Seed yields.
Data for partially cleaned seed (anticipating larger weight losses from contaminating debris) suggest that the 1015 parent in each cross gave 1.65 g/plant while the 1001 parent of HVHB4 (1053) yields only 0.45g and the 1001 parent of HVHB5 (1054) gave only 0.13 g. To put results in perspective 7.5 g / plant was considered a very achievable figure. Partially cleaned seed has been made available to Frontier and it is assumed this might deliver approximately 360 g of seed for them.

Seed viability.
Germination tests by NIAB showed that potential germination rates are good (97%+). Data on actual seed numbers per gram are awaited from NIAB.

Repeat summer seed production trial.
This will be half the scale of the winter production with 80 plants each from the 1015 and 1001 parents focusing almost entirely on the HVHB4 (1053) cross. While pollination and seed maturation will again have to be carried out in Long Days (space and technical constraints), a few plants will be retained in Short Days as a look-see into the interaction of day length at different stages.

Results from the 2005 project NFC0613 suggest that seed production will be much higher using the better light and higher temperatures possible under semi natural condition.

4.5 Agronomy – Led by Frontier

The establishment of all the locations was very poor due to the very dry condition in the summer of 2006. Germination was very uneven but appeared to better on compacted areas where seed contact with the soil moisture was best. Consequently several of the sites had to be abandoned.

Analysis of the seed at NIAB showed that the germination potential was only about 80% and that germination could occur over a significant period of time. This contrasts with our own UK commercial production (winter 2006/07) where the seed was both larger and had a greater than 95% level of germination.

Both the sown and hand transplanted material at NIAB grew very slowly during the hot dry conditions of June and July. Only in August when it started raining did the plant start growing rapidly such that a significant crop had been achieved by late September.

Figure 4 Mature plants from drilled seed.

Due to the non typical growing conditions and poor quality seed the herbicide trials were very inconclusive and are being repeated in 2007.

In 2007 this work has been repeated using higher quality seed supplied by Humber VHB. Due to the unpredictable growing conditions significant effort is also being made to establish crops using vegetable industry transplantation methods.

5 Conclusions

- High quality UK seed production is clearly feasible and with experience the efficiency of production can be greatly improved.
- Agronomically acceptable plant material with high artemisinin content has been identified and used to produce seed for UK production. Progeny testing will be possible in the summer of 2007.
- Significant development of the artemisinin analytical techniques has been possible and will make the effective selection in 2007 of improved material for UK production more accurate.
- The methods to be used for the field establishment of commercial crops has as yet to be proven.
- It will be possible to carry out fully replicated trials in 2007 of both the crosses and the parent material which will give a greater confidence in the results.

6 Technology transfer

As yet no significant effort has been made to do technology transfer as the current experimental work is as yet inconclusive. However significant effort is being made to develop the crop with input from farmers and vegetable growers through Frontier and seed production with Humber VHB.

7 Glossary

Chromophore:	light-absorbing moiety
Gradient:	Of chromatography: when a solvent or solvent mixture is gradually changed over time to elute a number of compounds from a column (e.g. starting with 100% water at Time 0, and finishing with 100% methanol at Time 1).
HPLC-DAD:	high-performance liquid chromatography-diode array detection
HPLC-MS (APCI):	high-performance liquid chromatography-mass spectrometry (atmospheric pressure chemical ionization)
HPLC-MS (ESI):	high-performance liquid chromatography-mass spectrometry (electrospray ionization)
IAA:	Indoleacetic acid
Isocratic:	Of chromatography: when a single solvent (e.g. 100%) methanol) or solvent mixture (e.g. 60% methanol, 40% water) is used to elute a number of compounds from a column
Maceration:	The extraction of herbal material by steeping in solvent
N:	theoretical plates (a measure of a chromatographic column's performance)
TLC:	thin layer chromatography

8 References

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9 Appendices

9.1 Response factor

Response factor of artemisinin with: Phenomenex RP-C18 250 mm x 4.6 mm (5 µm), using Acetonitrile 60%: H_2O 40% (1ml/minute isocratic) as mobile phase. Reference solution: Artemisinin in acetonitrile.

Means of calculation:
If reference solution contains 5 mg/ml artemisinin, then this is equivalent to 5000 µg/1000 µl of artemisinin.
5000/1000 = 5 µg/µl artemisinin in reference solution.
Since the injected volume of reference solution is 10µl, then within that injected volume are

5 x 10 = 50 µg artemisinin.

Assume this injected volume gives rise to a peak of size 2600 mAU*s (these are arbitrary units of UV absorbance). To determine how many mAU*s a single µg of artemisinin is responsible for, divide 2600 by 50.

2600/50 = 52.

Therefore 1 µg of artemisinin gives rise to 52 units of UV absorbance.

This information can be used to calculate the amount of artemisinin in an unknown sample. If, for example, the unknown shows a peak, at the same retention time and with the same UV profile as artemisinin, of 1500 mAU*s, then to find how much artemisinin is in that sample two steps are followed:

1. Divide the peak area by 52; that gives a value of (1500/52) = 28.9. Therefore there were 28.9 µg of artemisinin in the injected volume - say, 10 µl - of sample.
2. To obtain the amount of artemisinin in the total volume of sample, simply multiply 28.9 µg to the correct exponent. If the total sample volume was 1ml (or 1000µl), then multiply 28.9 by 100 to give (28.9 x 100) = 2890 µg (or 2.89 mg) in the unknown sample.

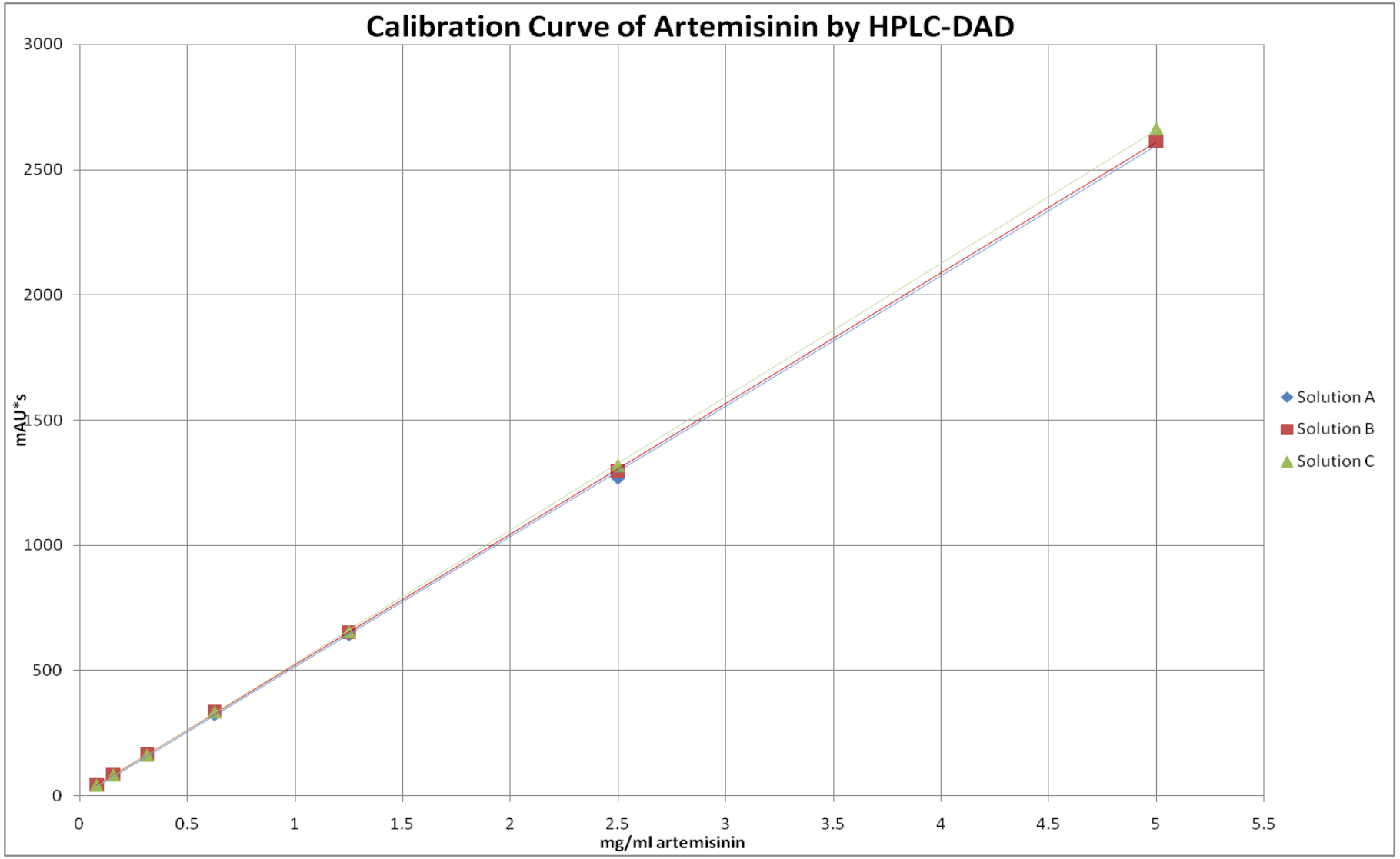
Note: if the flow rate or solvent composition of the method is changed, or if a gradient method is used, then the response factor must be re-calculated.

Table 6. Response factor as calculated on 29th July 2007:

mg/ml	*Solution*	*R1*	*R2*	*R3*	**Mean**	**SD**
0.078	*a*	51.4	52.06	52.1		
	b	52.78	52.41	53.14		
	c	52	52.37	52.25	**52.27889**	**0.493595**
0.15	*a*	51.3	52.38	51.65		
	b	52.85	53.25	53.02		
	c	52.2	52.03	51.78	**52.27333**	**0.661362**
0.3	*a*	51.16	51.25	51.23		
	b	52.97	52.73	52.58		
	c	51	50.9	51	**51.64667**	**0.848351**
0.6	*a*	50.52	50.77	50.53		
	b	53.06	53.01	53.3		
	c	52.25	52.4	52.64	**52.05333**	**1.134923**
1.2	*a*	50.19	50.46	50.47		
	b	51.49	51.39	51.39		
	c	51.57	51.26	51.22	**51.04889**	**0.523485**
2.5	*a*	49.72	49.67	49.77		
	b	51.15	50.97	51.14		
	c	51.8	51.69	51.67	**50.84222**	**0.887705**
5	*a*	51.12	51.11	51.31		
	b	51.5	51.56	51.46		
	c	52.35	52.15	52.16	**51.63556**	**0.468111**
	Sum of Means:	**51.65583**				

R: replicate injection. Three stock solutions (A, B and C) were prepared, of 5 mg/ml. Each of these was serially diluted to obtain the reference solutions described in yellow columns. Each reference solution was injected three times to obtain the values in the green columns.

Figure 5 Calibration Curve of Artemisinin by HPLC-DAD



Conc. artemisinin (mg/ml)	Symmetry	Peak Width	Plate Count†
0.078	0.87	0.1633	66315
0.15	0.86	0.1633	65823
0.31	0.85	0.1650	64075
0.625	0.82	0.1650	63991
1.25	0.77	0.1667	62946
2.5	0.69*	0.1689	60652
5.0	0.56*	0.1689	56224

† Samples were analysed in the order: least concentrated →most concentrated, over a 24 hour period. Therefore the drop in *N* may reflect column conditions deteriorating slightly throughout the sequence, rather than being a function of sample concentration.

* tailing