PROJECT REPORT

EXTRACTION OF ARTEMISININ USING IONIC LIQUIDS

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003-003/3

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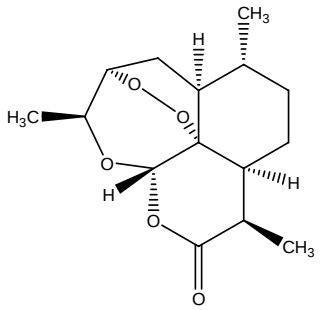
1. Summary

This report covers a multi-component project evaluating the technical issues concerning the use of ammonium-based ionic liquids as solvents for the extraction of artemisinin. The previously studied solvent N,N-dimethylethanolammonium octanoate was further evaluated for this application, with particular emphasis being placed on the elucidation of its extraction profile and the issues associated with the recovery of artemisinin from solution. Results from these studies were subsequently used to design an iteratively improved solvent, which was similarly evaluated in order to establish key design parameters for a solvent geared for optimal extraction performance. The results reported herein delineate a template for the development of a commercially viable ionic liquid to address these requirements.

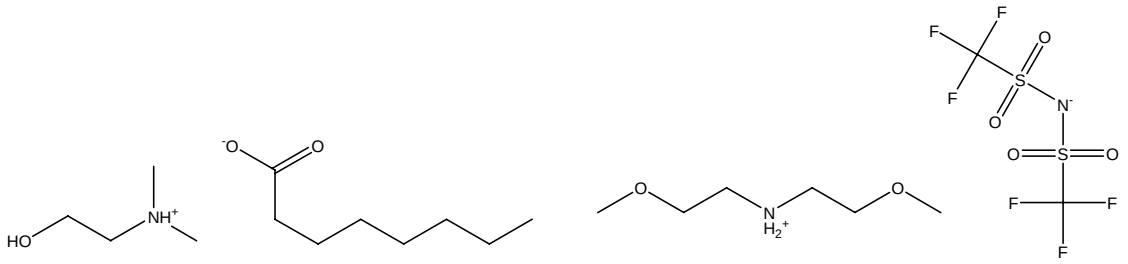
2. Introduction and aims

2.1 Previous work



Artemisinin (**1**)

Previous studies performed by Bioniqs Ltd. evaluated the efficacy of a small range of proprietary ionic liquids as alternative media for the extraction of the antimalarial agent artemisinin (**1**) from *Artemisia annua*.[1,2] These solvents were selected from a stock product range on the basis of evidence from *in silico* simulation studies of their interactions with pure artemisinin. Four solvents were selected and all were experimentally evaluated for the extraction process, both with respect to each other and versus *n*-hexane when used under identical conditions. This evaluation was extended to include an indirect comparison with hydrofluorocarbon (HFC) solvents, supercritical carbon dioxide and ethanol.[3] Of the ionic liquids tested, the most satisfactory preliminary results were obtained using N,N-dimethylethanolammonium octanoate (DMEA oct, **2**) and bis(2-methoxyethyl)ammonium bis(trifluoromethanesulfonyl)amide (BMOEA bst, **3**).



N,N-Dimethylethanolammonium octanoate (DMEA oct, **2**)

Bis(2-methoxyethyl)ammonium bis(trifluoromethanesulfonyl)amide (BMOEA bst, **3**)

The fluorinated solvent **3** exhibited an higher overall recovery of artemisinin and a greater stability of the extract once in solution than was the case using **2**; however, the combined advantages of biodegradability and more rapid product extraction resulted in DMEA oct being preferred. Subsequent studies into the use of this ionic liquid established the optimal extraction conditions to comprise stirring at room temperature at a solvent:leaf ratio of 3:1 w/w. Problems were however encountered in terms of the recovery of artemisinin from solution; this was initially accomplished with considerable success by the addition of water, resulting in the precipitation of artemisinin, which was recovered by filtration. Subsequent attempts to reproduce this methodology using extracts from additional batches of plant evidenced considerable instability in the system, resulting in the artemisinin precipitating from solution in variable forms ranging from isolable crystals through finely dispersed flocculent suspensions to emulsions. The finer precipitates could not be satisfactorily recovered from solution, hence prevented ionic liquid **2** from being suitable for in field application.

It was recommended that additional work should be performed in order to ascertain the source of this variability and thence to modify the chemical structure of the ionic liquid, the ultimate aim being a substantial improvement in all of the requisite properties necessary for effective extraction of artemisinin. The described project was undertaken to address this overall objective, manifested as the series of specific aims described below.

2.2 Aims

The following set of aims was designed to ascertain whether varying levels of co-extracted impurities present in the pre-precipitation ionic liquid extract were likely to interfere with the precipitation and recovery process and thence to investigate whether structural modifications to the ionic liquid could improve performance in this regard. To this end, exhaustive extractions of a comparatively large batch of *A. annua* were performed using both chloroform and **2**. The ranges of individual compounds present in these extracts were then compared and the key unknown impurities present in the ionic liquid extract were purified and identified. These compounds were then modelled *in silico* in

the context of the precipitation environment, in order to ascertain any likely effect upon the precipitation. The specific experimental objectives were therefore:

1. to evaluate the impurity profile for extractions of *Artemisia annua*, involving exhaustive extractions with cold chloroform, analytical and semi-preparative high performance liquid chromatography (HPLC) with fraction collection and analyte identification by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and mass spectrometry.
2. to repeat the established extraction methodology using N,N-dimethylethanolammonium octanoate on plant material of defined water content and composition (as found by chloroform extraction), with artemisinin quantification and extractant profiling by HPLC with refractive index (RI) detection.
3. to investigate the recovery of artemisinin from N,N-dimethylethanolammonium octanoate extracts by addition of water and any dependence of precipitation efficiency upon the presence of water and any defined co-extractants identified from the steps above.
4. to model the likely contribution of co-extracted impurities upon the water/IL/artemisinin precipitation balance.
5. to modify the structure of N,N-dimethylethanolammonium octanoate in an iterative and incremental fashion, to enhance extraction selectivity, solution phase life time of artemisinin and reproducibility and robustness of the precipitation method.
6. to manufacture and purify one or more incrementally improved ionic liquid(s).
7. to repeat the extraction protocol using the improved ionic liquid(s) on various batches of plant material and to evaluate their relative efficacy.
8. to conduct a basic microscopy study into the effects of ionic liquid exposure upon trichome cells and comparison with effects elicited by chloroform.
9. to investigate the effect of various mixing techniques and plant material size parameters upon extraction yield and efficiency.
10. to evaluate methods for the recovery of artemisinin from solution, in sufficient yield and purity for subsequent processing, including evaluation of solvent carry-over.
11. to quantify residual solvent contamination of exhausted biomass and form an opinion on likely resulting effects upon treatment and disposal strategies.

3. Materials and methods

3.1 Materials

Ionic liquids were synthesized in house to analytical specification, according to established internal procedures. Precursor amines and acids were sourced from Sigma Aldrich UK Ltd., Gillingham, UK or were custom manufactured by Almac

Group, Belfast, UK. Precursors were utilized at greater than 99% purity and were if necessary distilled prior to use. Ionic liquids were analyzed by ion chromatography and Karl Fischer titration and were ≥99% pure with a water content of ≤0.5% at point of use. Solvents used were HPLC grade and were sourced from Fisher Scientific UK Ltd., Loughborough, UK. Artemisinin was kindly supplied by Medicines for Malaria Venture (MMV), Geneva, Switzerland. The generous donation of dried *Artemisia annua* leaf by Dr, Xavier Simonnet of Mediplant, Conthey, Switzerland is gratefully acknowledged. Any additional specialist items or reagents were sourced as described in the text of the relevant section of this report.

3.2 Methods

3.2.1 Extraction methods

Extractions were performed according to the method previously employed, in order to ensure comparability.[2] Dried *A. annua* leaf (3.0g) was used in all extractions, with the volume of the solvent being varied to achieve the desired ratio.

Extractions were performed at room temperature with continuous magnetic stirring, or manual agitation in cases where the viscosity or solvent:leaf ratio of the sample prevented effective stirring, for 10 minutes. Ionic liquid extractions were performed in duplicate and were worked up by pressing through a muslin filter using a modified syringe, followed by micro-filtration through a 0.2μm syringe filter and centrifugation at 4990rpm for up to 20 minutes.

The exception to this was the exhaustive extraction with chloroform, in which 100g dried *A. annua* was extracted with 700mL of solvent under otherwise identical conditions. The biomass was removed by vacuum filtration prior to removal of the solvent *in vacuo* and analysis as described in the appropriate section.

3.2.2 Analytical methods

3.2.2.1 High performance liquid chromatography (HPLC)

The International Pharmacopoeia monograph for artemisinin describes quantification by means of HPLC with ultraviolet spectroscopic detection of the analyte.[4] Despite this, the literature maintains that pure artemisinin does not absorb in the ultraviolet region, a fact we have confirmed experimentally.[5] As a consequence, we have developed and validated an alternative HPLC method for the quantification of artemisinin, using refractive index (RI) detection, a protocol for which has been made available.[6] This method was utilized for all HPLC analyses performed in the work described herein.

Chromatography was performed using a Dionex Ultimate 3000 HPLC system running a 250x4.6mm Phenomenex Gemini 5μ C18 110Å reverse phase column. The mobile phase used was 60:40 acetonitrile:water, which was degassed prior to use. Samples (200μL) were prepared for injection by dilution into mobile phase at a concentration of 1-4mg/mL; these samples were then pre-mixed in a 1:1 ratio with an internal standard (β-artemether, Apin Chemicals Ltd., Abingdon, UK) prepared in mobile phase at 2.5mg/mL, filtered through a 0.2μm syringe filter and degassed. An injection volume of 25μL was used in all samples. For artemisinin quantification alone, a flow rate of 1.0mL/minute was adopted, with a run time of 20 minutes; under these conditions, artemisinin eluted after a retention time of 9-9.5 minutes. For semi-preparative and extraction profiling studies, better separation was achieved by reducing the flow rate to 0.5mL/minute over a 30 minute run time, with artemisinin then eluting after approximately 20 minutes.

Detection was elicited using a Shodex RI-101 refractive index detector. Data were processed using Chromeleon software and quantification was achieved by comparison with a standard curve produced using pure artemisinin (**Figure 1**), at a concentration range of 0.05-20mg/mL.

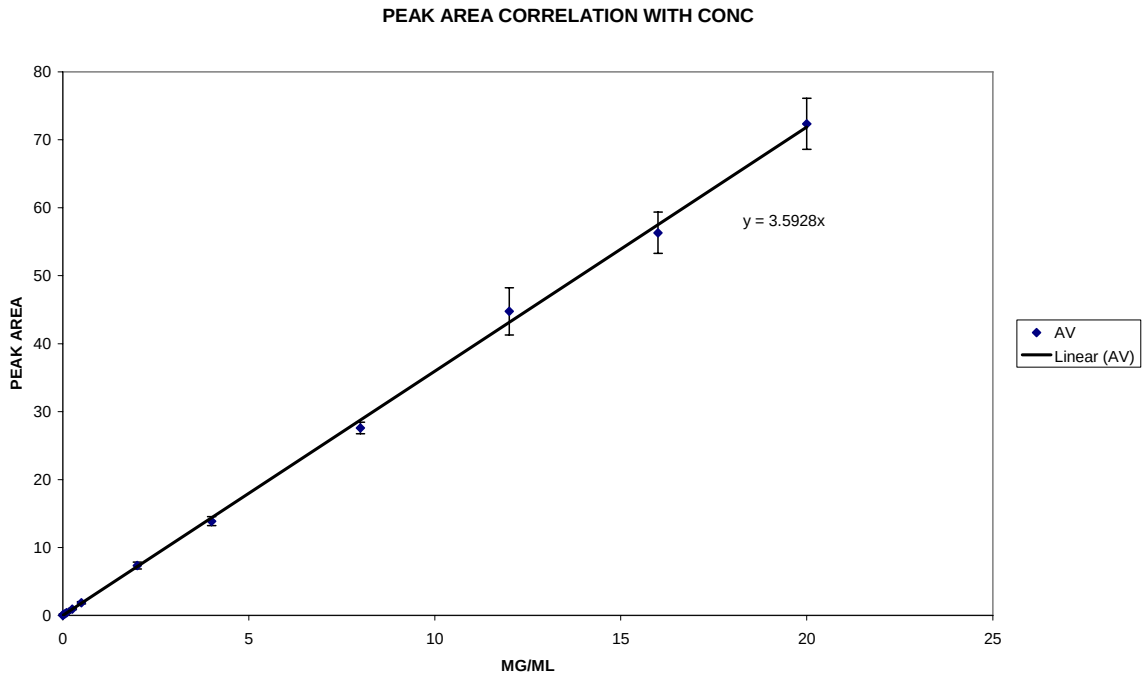


Figure 1. HPLC-RI calibration curve for artemisinin

Semi-preparative HPLC was performed under identical conditions but using a 250x10mm column and correspondingly larger loop. Eluted fractions were collected automatically using a Teledyne Isco Foxy Junior fraction collector.

3.2.2.2 Ion chromatography

Ion chromatography was utilized in order to quantify both the purity of the ionic liquids used and the level of residual solvent remaining in the recovered product and exhausted biomass post extraction. Analysis was performed using a Dionex ICS3000 ion chromatograph, running a 250x9mm Dionex IonPac ICE-AS1 column with 1mM perfluorobutyric acid eluent for anions and a 250x3mm Dionex IonPac ICE-CS18 column with 5mM methanesulfonic acid eluent for cations. Flow rate was 0.8mL/min in both cases. Samples (1mL) were injected manually, following dilution in the mobile phase to an appropriate concentration for comparison with the standard curve for the ion in question.

3.2.2.3 Nuclear magnetic resonance (NMR) spectroscopy

Proton (^{1}H) NMR spectra were recorded using a Jeol 270MHz instrument; samples were prepared in 0.5mL $CDCl_3$, with tetramethylsilane as the internal reference standard. FID spectra were processed using NutsNMR software.

3.2.2.4 Mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) was performed at CNAP, Department of Biology, University of York., using a Waters Acquity liquid chromatography system coupled to a Thermo LTQ Orbitrap mass spectrometer. An acetonitrile:water gradient was run in the presence of 0.1% formic acid to aid ionization. Separation was performed on a 2.1x50mm Phenomenex Luna HST C18 column.

3.2.2.5 Water content determination

Water content of both solvents and biomass was measured using a KEM MKS-500 volumetric Karl Fischer titrator with Methanol Rapid solvent and Hydranal Composite 5 titrant.

3.2.2.6 Residual solvent analysis

Residual ionic liquid levels were assayed by ion chromatography as described above. Analyte samples (500mg) were steeped in the appropriate ion chromatography eluent (100mL) for 15 minutes at room temperature, after which the solution was filtered through an 0.2μm syringe filter prior to injection. The concentrations of liberated ions were then quantified by comparison with the relevant standard curve and the weight % of ionic liquid present in the original analyte sample was calculated.

3.2.2.7 Microscopy

Microscopy was performed at CNAP, Department of Biology, University of York, using a Leica MZ6 instrument at 64x magnification. Basic procedures for qualitative trichome studies were taken from the literature.[7]

3.2.3 Molecular modeling

Molecular simulation studies were performed within the framework of the Bioniqs proprietary database.

4. Results

4.1 Evaluation of chloroform extract

Coarse-milled *A. annua* leaf supplied by Mediplant was analysed by Karl Fischer titration, as described above. The water content of the material was thereby established to be 29.8 $\pm$ 0.5%. Plant biomass was stored as received in tied plastic bags, placed in a closed cupboard. Subsequent ongoing analyses determined the water content to rise to 31.5 $\pm$ 3.5%. Leaf material (100g) was extracted with chloroform (700mL) under the conditions outlined in Section 3.2.1. The solvent was removed *in vacuo* to yield 11.6g of a greenish semi-crystalline solid residue. This was redissolved in a minimal volume of 50:50 diethyl ether:40-60 petroleum ether and manually chromatographed on a 30x2cm glass column packed with 60Å silica. Components were eluted using a 40-60 petroleum ether/diethyl ether/chloroform/methanol gradient. 120 fractions were collected; each was then stripped of solvent in vacuo, resuspended in 60:40 acetonitrile:water, sonicated for 5 minutes, filtered through a 0.2μm syringe filter and analysed by HPLC-RI as previously described.

The number of individual compounds potentially present in an exhaustive chloroform extraction of *A. annua* is vast;[7,8,9] hence the objective of the study was not to identify every single component but to isolate individual compounds on the basis of their retention time under the HPLC conditions employed. Analysis indicated the presence of artemisinin at a concentration equating to 1.45% by weight of the plant material, calculated from the HPLC trace shown in **Figure 2b** by comparing the area of the artemisinin peak against the standard curve shown in **Figure 1** to determine the concentration of artemisinin in mg/mL in primary extract, then calculating the weight % of the original plant biomass, which this value represented. As anticipated, a large number of co-extractants were also present with retention times of from 3 to 27 minutes. Owing to the fact that standards of the known components of *A. annua* were not available, it was not possible to construct calibration curves or therefore to accurately quantify the relative levels of these impurities; however, the chromatogram (**Figure 2b**) showed the presence of six major co-extractant peaks with retention times of 5.1, 9.4, 9.8, 13.2, 14.1 and 17.1 minutes, representing highly refractive and/or highly concentrated materials. Fractions were separated and collected according to

the previously described protocol and were stored at -20ºC whilst the ionic liquid extractions were carried out.

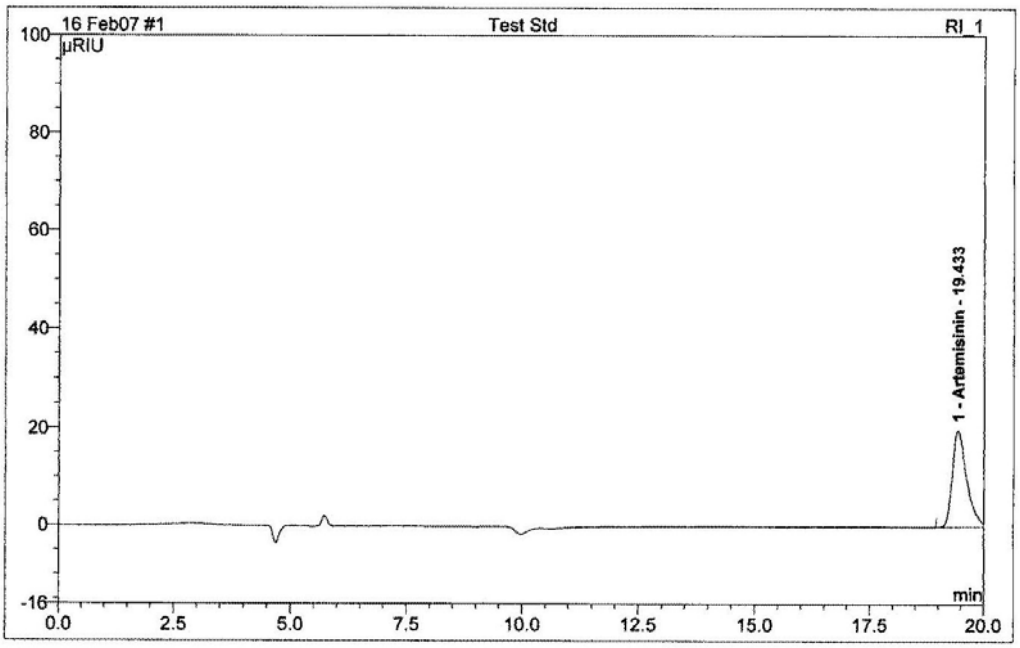
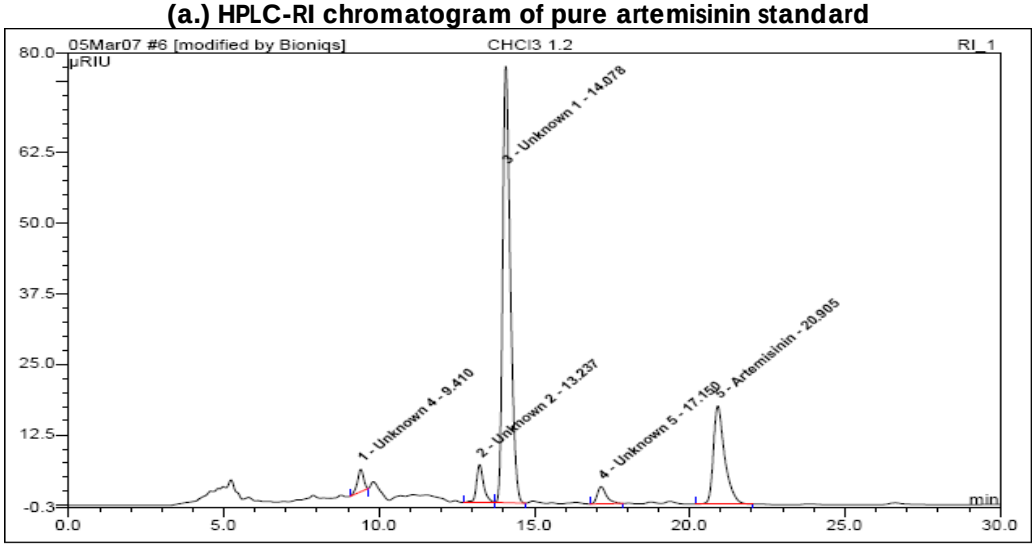


Figure 2
(a.) HPLC-RI chromatogram of pure artemisinin standard



No.	Ret.Time min	Peak Name	Height µRIU	Area µRIU*min	Rel.Area(ident.) %	Amount mg/l	Type
1	9.41	Unknown 4	3.954	0.905	2.73	0.122	BMB*
2	13.24	Unknown 2	6.704	1.778	5.36	0.239	BMB
3	14.08	Unknown 1	77.258	22.113	66.63	2.977	BMB*
4	17.15	Unknown 5	3.056	1.032	3.11	0.139	BMB
5	20.91	Artemisinin	17.409	7.359	22.18	0.991	BMB
Total:			108.381	33.187	100.00	4.468	

Figure 2
(b). HPLC-RI chromatogram of primary *A. annua* extract using chloroform, showing presence of artemisinin (far right) and 6 major co-extracted impurities, with retention times of 5.0, 9.4, 9.8, 13.2, 14.1 and 17.1 minutes respectively.

4.2 Evaluation of N,N-dimethylethanolammonium octanoate extract

Extraction of *A. annua* by DMEA oct (**2**) was performed according to the described method using a solvent: leaf ratio of 2:1 w/w. HPLC analysis confirmed that results were similar to those observed in previous studies in terms of the amount of artemisinin extracted.[1,2] Artemisinin was present at a concentration equal to 0.36% of the dry weight of the plant. When this ratio was increased to 7:1, the amount of artemisinin extracted was equivalent to 0.45-0.47% by weight of the dried plant. The HPLC chromatograms for extractions using this ionic liquid were impaired by the long tailing of the octanoate anion eluting from the column (**Figure 3**), thus the identification of minor impurities was not possible under the HPLC conditions used; however, only 3 major impurities were extracted by N,N-dimethylethanolammonium octanoate compared to the 6 observed with chloroform. These major impurities corresponded to those seen with chloroform at retention times of 5.0, 9.4 and 14.1 minutes respectively.

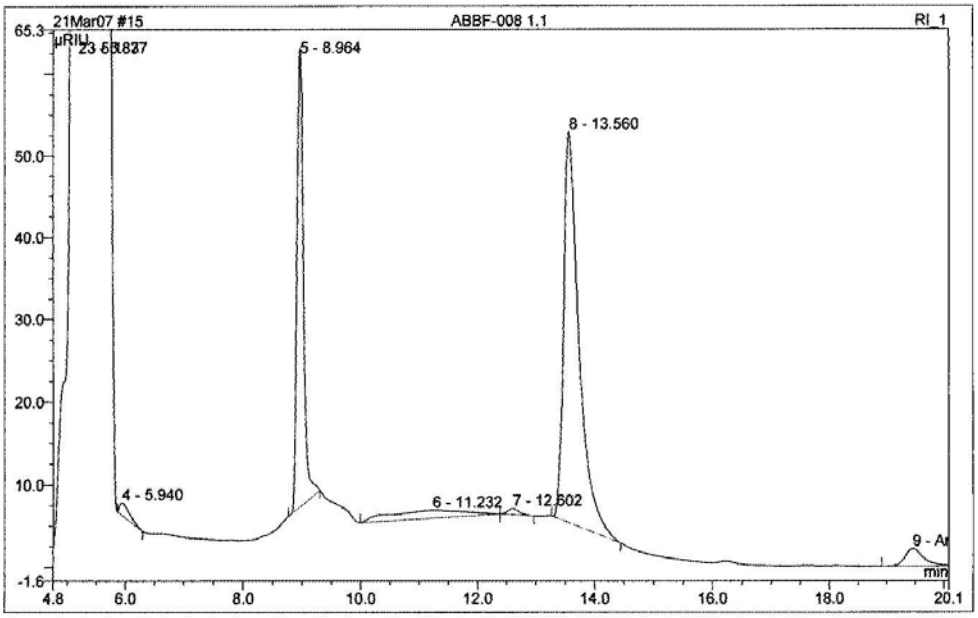


Figure 3.
Specimen HPLC-RI chromatogram of DMEA oct primary extract showing tailing of ionic liquid peak

To confirm the identity of the co-extractants present in the primary DMEA oct extract, the chloroform extract samples with identical retention times were removed from the freezer and spiked into the ionic liquid extracts. Re-analysis confirmed that the peak size was increased proportionally (**Figure 4**).

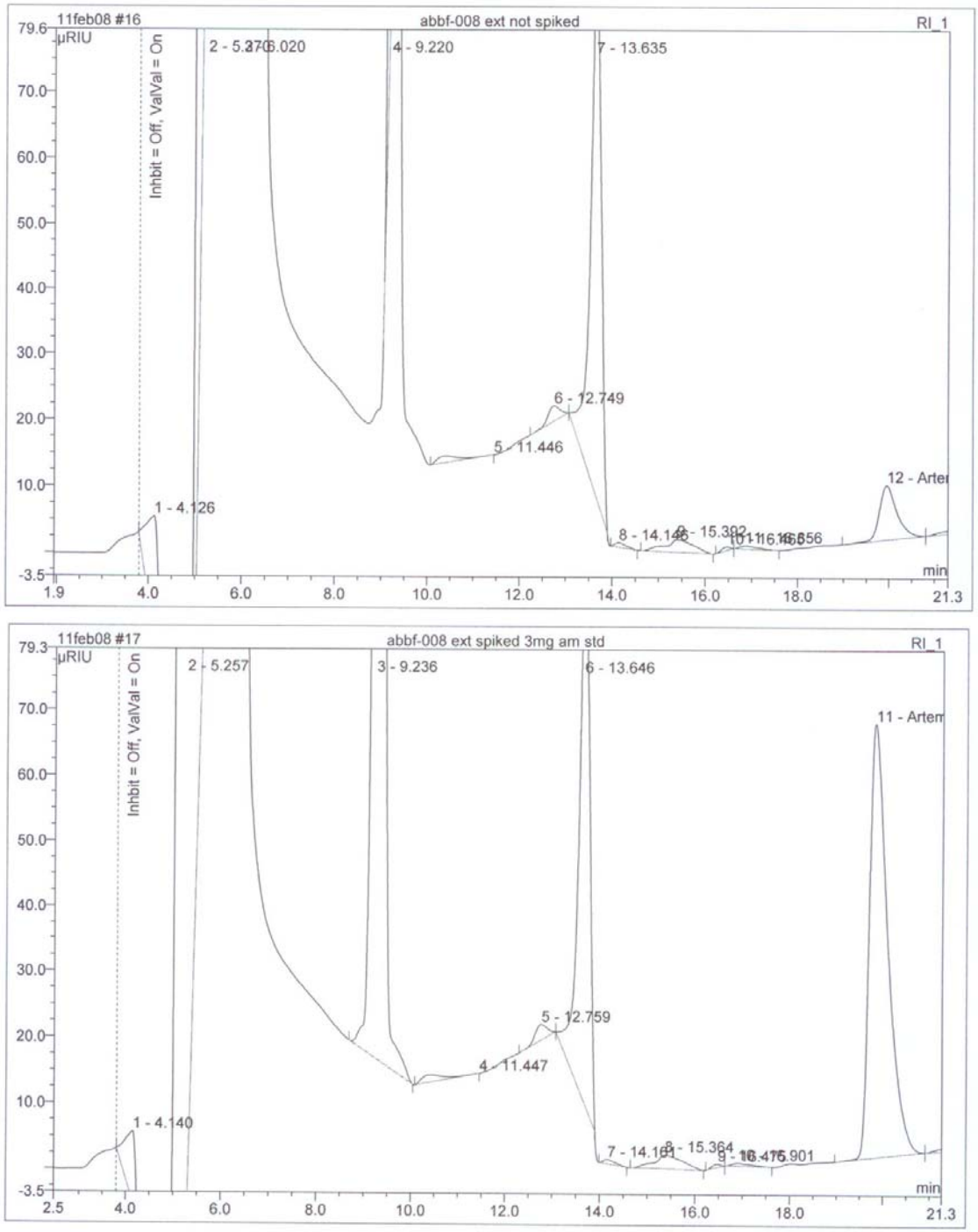


Figure 4.
HPLC-RI chromatograms of DMEA octanoate primary extract confirming identity of artemisinin peak by adding 15mg/mL pure artemisinin to the primary extract. Figure shows HPLC trace before (top) and after (below) addition.

Having established commonality, the chloroform extract samples were submitted for NMR and mass spectrometry in an attempt to establish their

chemical structures and identities. Four samples were analysed; those with HPLC retention times of 5.0, 9.4, 14.1 and 20.9 minutes (the latter believed to be artemisinin). The area of this latter peak equated to 3.6% of the sum of the extractant peak areas; however given that the other extractants were of unknown identity and hence no standards could be run to quantify their refractive index response, this value is NOT representative of the proportion of the extracted component by mass made up of artemisinin.

a. sample with retention time of 5.0 minutes

Analysis of the compound eluting after 5 minutes was inconclusive. The mass spectrum (**Figure 5**) showed the presence of two major peaks with molecular weights of 283 (isomeric with artemisinin) and 237, suggesting a terpenoid structure; however, the retention time of this compound was one quarter that of artemisinin, implying considerably higher polarity. The fragmentation pattern observed in the mass spectrum was also distinctly different to that for artemisinin or to any of the published patterns for other related terpenoids known to be present in the plant (arteannuin B, artemisinic acid and the corresponding dehydro-forms). The NMR spectrum was similarly inconclusive. In order for this compound to be accurately identified, additional analysis must be performed.

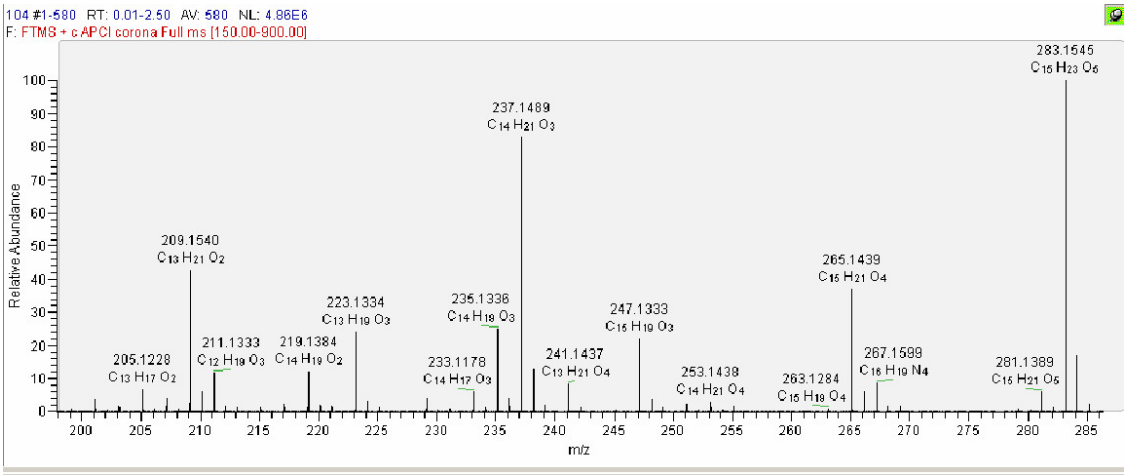
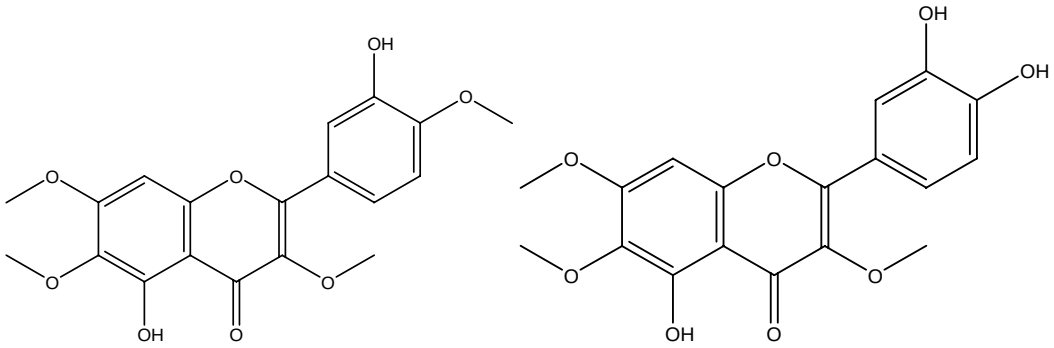


Figure 5. ^{1}H NMR spectrum of unknown co-extracted impurity with HPLC retention time of 5.0 minutes.

b. samples with retention times of 9.4 and 14.1 minutes

The mass spectra of these compounds were relatively clean and gave molecular weights of 361 (**Figure 8**) and 375 (**Figure 6**) respectively. These are in the typical region for flavonoids, representing trihydroxytrimethoxyflavone ($C_{18}H_{16}O_8$) and dihydroxytetramethoxyflavone ($C_{19}H_{18}O_8$) respectively. The latter compound was present in significantly greater amounts and represented the major co-extracted impurity present in the DMEA oct extract. Analysis of the NMR

spectrum of this material (**Figure 7**) showed 4 singlets at δ values between 3.7-3.9, a singlet at δ=6.43, a doublet at δ=6.99 and a double doublet at δ=7.64. Comparison with the literature confirmed these spectra as characteristic of casticin (3',5-dihydroxy-3,4',6,7-tetramethoxyflavone, **4**), a known significant component of the aerial parts of *A. annua*.[8,10] The NMR spectrum of the trihydroxytrimethoxyflavone (**Figure 9**) was insufficient to be able to precisely locate the demethylated oxygen atom relative to casticin, but a structure isomeric to that shown in **5** can be assigned. Flavonoids therefore composed by some distance the major impurities present in the DMEA oct extract.



Casticin (**4**) Trihydroxytrimethoxyflavone (**5**)

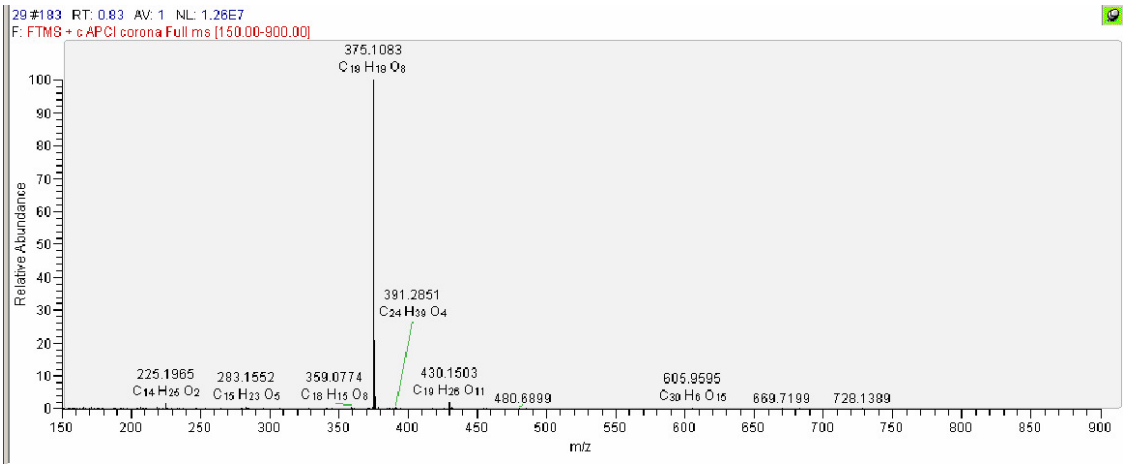


Figure 6. Mass spectrum of casticin (4)

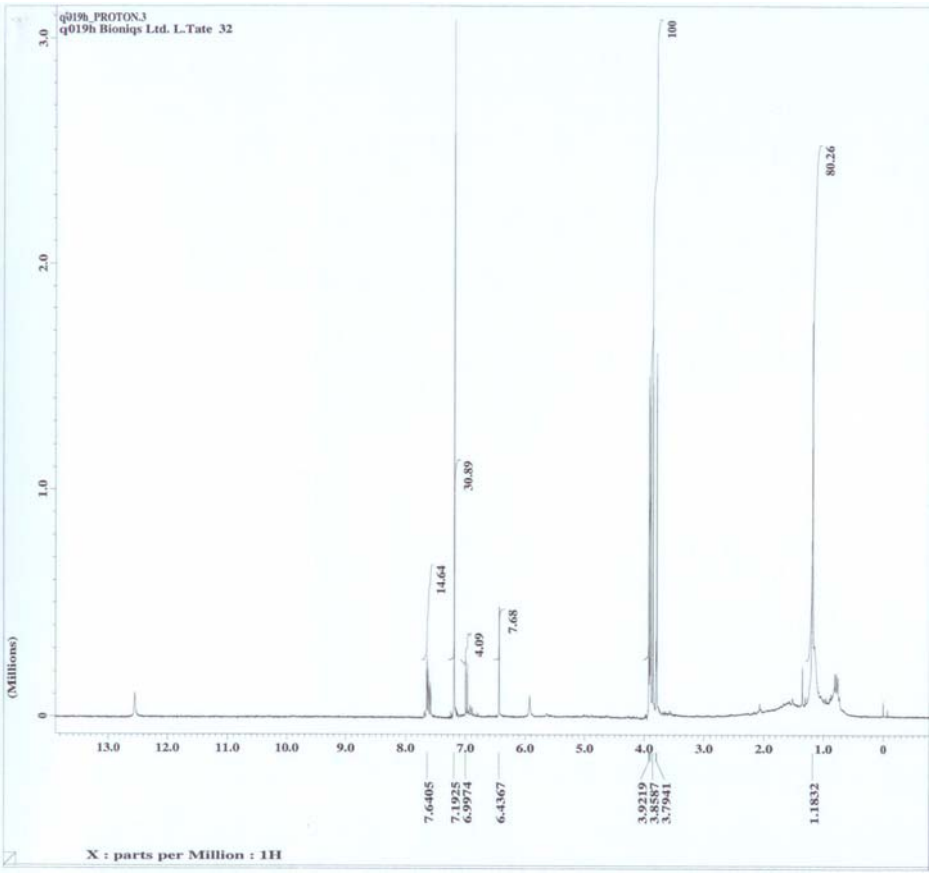


Figure 7. ^{1}H NMR spectrum of casticin (4)

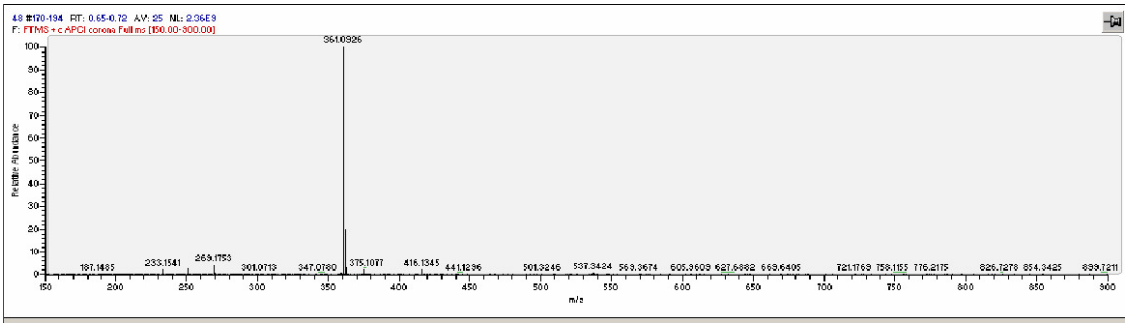


Figure 8. Mass spectrum of trihydroxytrimethoxyflavone (5).

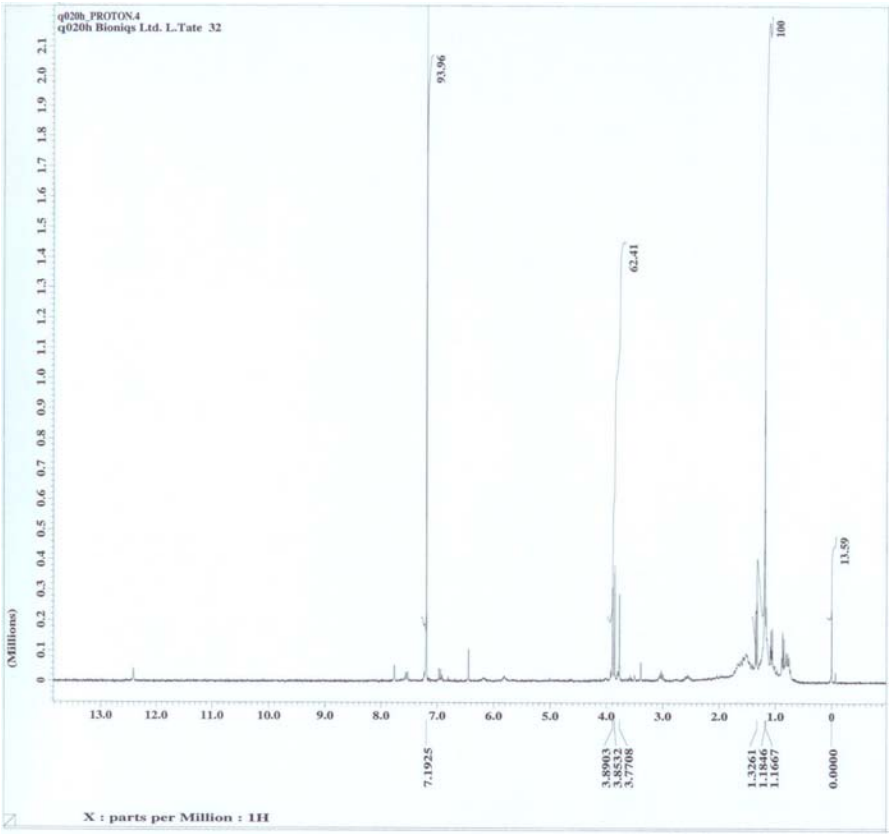


Figure 9. ^{1}H NMR spectrum of trihydroxytrimethoxyflavone (5).

c. sample with retention time of 20.9 minutes

This compound was confirmed as artemisinin (**1**) by comparison of its mass (**Figure 10**) and NMR (**Figure 11**) spectra with the literature.[11] Notable within the mass spectrum was the large peak at M_r=565, apparently resulting from dimerization (artemisinin M_r=283). This is believed likely to have arisen during the ionization within the mass spectrometer.

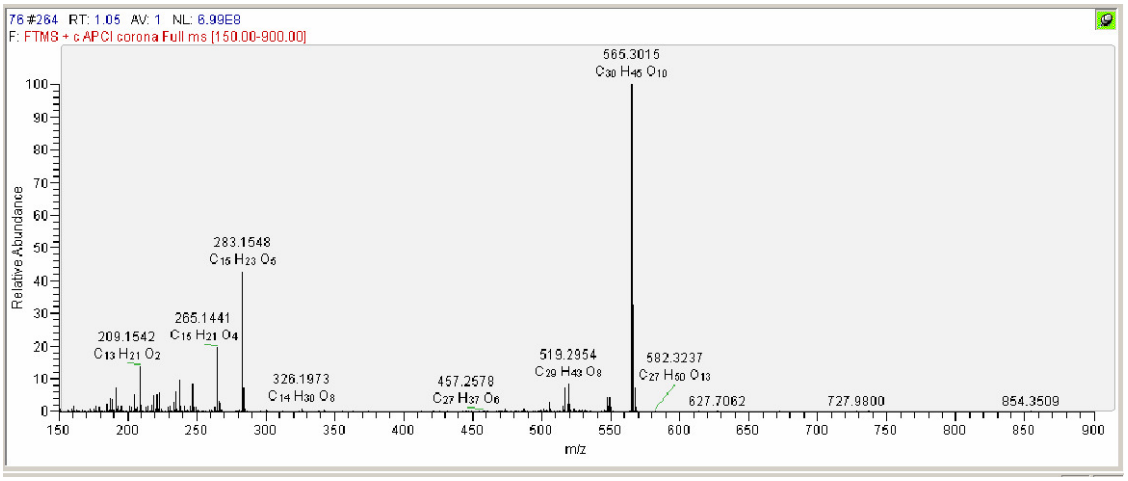


Figure 10. Mass spectrum of artemisinin (1)

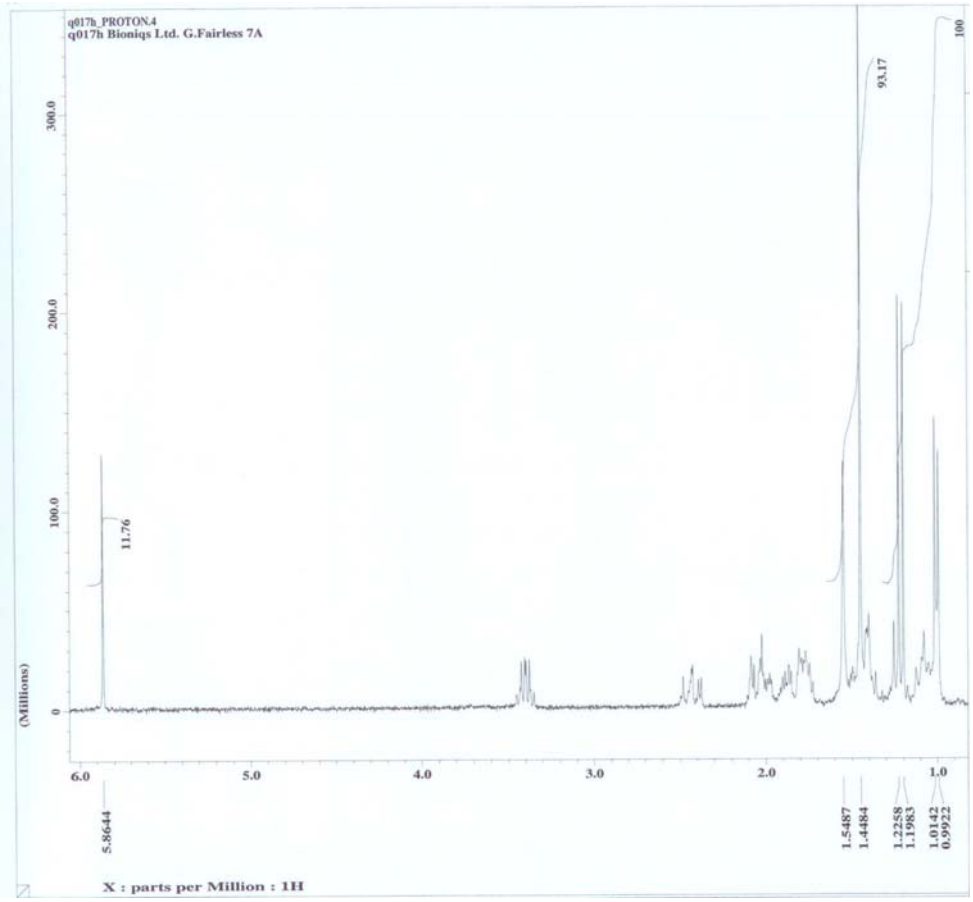


Figure 11. ^{1}H NMR spectrum of artemisinin (1) Note doublets at δ=0.9 and δ=1.2 (C6 and C9 methyl groups), multiplet at δ=3.4 (H9), singlet at δ=5.9 (H12), multiplet at δ=1.4 (C4, C5, C7 & C9 methylenes) and methine protons at δ=1.5 (C5a, C8a & C9)

4.3 Recovery of artemisinin from N,N-dimethylethanolammonium octanoate

Recovery of artemisinin from DMEA oct solution by the addition of water was previously achieved with considerable success;[1] however, the reproducibility of this process has subsequently been shown to be poor, with major variations in the particle size of the precipitate and a consequent inability to routinely recover by filtration.[2]

Recovery of precipitate from DMEA oct was achieved by dividing primary extract (50mL) into ten equal aliquots of 5mL and adding a defined volume of distilled water. The form of the precipitate was noted and it was then recovered by vacuum filtration through a silica bed over a sinter, followed by steeping the recovered silica in 60:40 acetonitrile:water, filtration to remove insoluble material and artemisinin quantification and composition profiling by HPLC.
The volume of water added to the DMEA oct extract varied from 0.5 to 100 equivalents. In all cases, addition of water elicited the precipitation of white flocculent solid, which could not be directly isolated but could be trapped on the silica bed and recovered by redissolution in mobile phase. The composition of this precipitate with respect to plant derived components was essentially pure artemisinin regardless of the volume of water added; the flavonoids remaining in the aqueous ionic liquid and not precipitating to any detectable extent at any dilution. Significant variations did however exist between the ten samples with regard to the proportion of the artemisinin recovered and the level of residual ionic liquid contaminating the isolated product, as shown in Table 1.

Volumes H_2O added	% artemisinin recovered	g IL per g artemisinin
0.5	45 +/- 10	62-85
1	59 +/- 5	40-67
1.5	66 +/- 8	36-58
2	75 +/- 6	30-50
5	82 +/- 5	15-35
10	80 +/- 8	8-27
25	69 +/- 5	4.5-18
50	55 +/- 5	1.0-7
75	47 +/- 10	0.6-1.5
100	34 +/- 8	0.2-0.8

Table 1. Recovery of artemisinin from DMEA oct by precipitation with water

In summary therefore, the precipitation of artemisinin from DMEA oct primary extract by the addition of water has some notable advantages, but also a number of drastic flaws precluding it from consideration as a practical system. The fundamental principle of the procedure is that, given the fact that both the co-extracted impurities and the ionic liquid itself are appreciably more polar than artemisinin and hence more soluble in water, the artemisinin should be the

only component of the solution to precipitate when water is added. This was borne out empirically; the greater the volume of water added, the more completely solubilized was the ionic liquid and the more effective the precipitation, up to the point where the volumes of water being added were so large that the sample was massively diluted and the aqueous solubility of artemisinin became significant. Reanalysis of the post-filtration aqueous supernatants from the above fractions confirmed that the flavonoids remained almost entirely in solution, along with the balance of the extracted artemisinin (**Figure 12**).

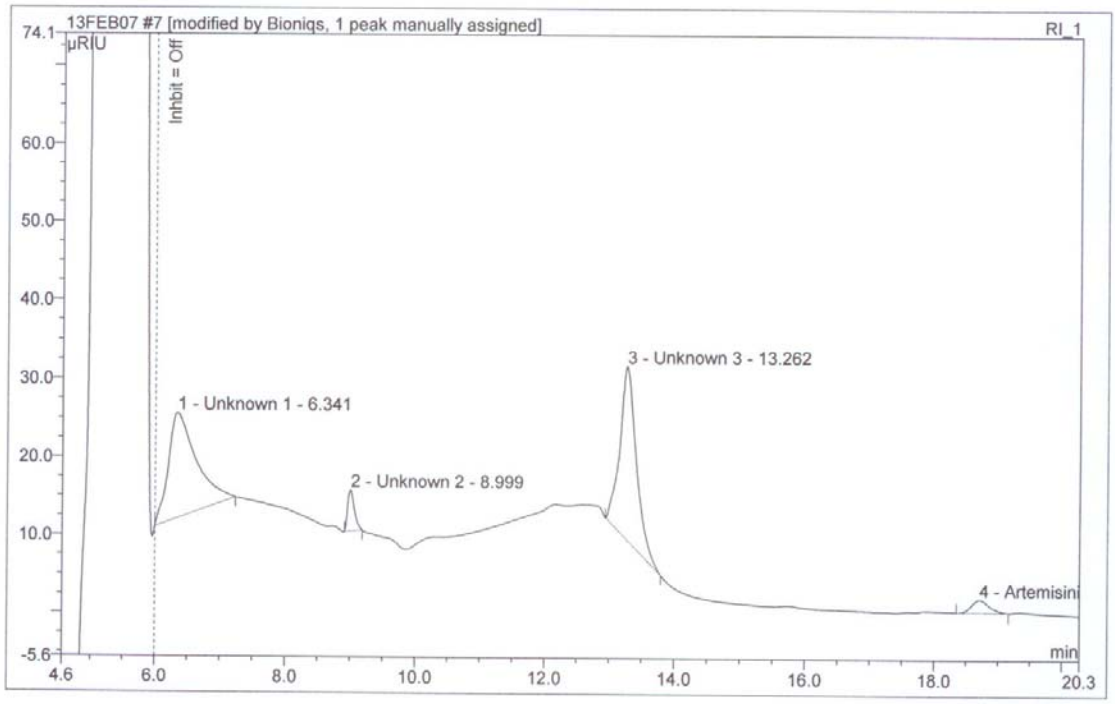


Figure 12.
HPLC-RI chromatogram of aqueous DMEA oct supernatant remaining after precipitation of the bulk of the dissolved artemisinin.

However, whilst artemisinin was the only extracted compound to precipitate significantly, the isolation and purification of the product was hampered by the very large proportional volumes of ionic liquid which were also detected in the "precipitated" material. To a large extent, these values are an artifact of the recovery process – the precipitate being too fine and flocculent in nature to be effectively filtered by conventional means. Numerous alternative methods of eliciting precipitation were attempted in order to try to force the artemisinin to precipitate in a more easily isolable form, including seeding with authentic artemisinin crystals, crash-cooling to 0°C, decanting, centrifugation and various different filter media, all without significant improvement. The use of the silica bed, whilst effective at removing the finely precipitated artemisinin, also served to trap large quantities of ionic liquid, particularly at lower dilutions. This ionic

liquid was then liberated into the HPLC mobile phase on standing, hence giving rise to results suggesting that the "precipitate" contained up to 99% by weight of ionic liquid. Washing the precipitate with water in an attempt to remove the bulk of the ionic liquid was attempted, but the volumes of water needed were so great as to elicit unacceptably high levels of artemisinin loss. The 99% figure is clearly not a valid reflection of the actual composition of the precipitated solid (a solution of 1% artemisinin in DMEA oct being completely liquid at room temperature); nevertheless, a more reasonable quantification of the amount of ionic liquid carried over would be dependent upon the development of a more efficient filtration system for the removal of an ultra-fine precipitate. Given the variability in the form of the precipitate and the likely recycling problems associated with the removal of large volumes of water and non-volatile casticin from the supernatant, it was considered far more practical to evaluate the potential for modifying the ionic liquid than to address the prohibitively complex chemical engineering issues surrounding the development of any kind of artemisinin extraction/recovery process using DMEA oct.

The objective was therefore set to utilize molecular modelling in order to construct a modified ionic liquid, retaining the same general structure as DMEA oct as a base and remaining equally selective for artemisinin in the presence of other terpenoids, but exhibiting a much lower affinity for casticin and a reduced surfactant effect, to prevent the ionic liquid from solvating artemisinin so strongly that it remains in solution even at substantial aqueous dilutions, thereby inhibiting complete and reproducible precipitation.

4.4 Residual solvent analysis

The amount of ionic liquid remaining adsorbed onto or absorbed into the spent post-extraction biomass is a critical factor from the perspective of both recycling the solvent with minimal losses and rendering the biomass safe for disposal. Unfortunately, the equipment utilized at bench scale in this study was however much too crude to permit any reasonable assessment of the solvent loss per cycle to be made.

Following extraction, the bulk of the solvent was removed by vacuum filtration; the remaining "wet" biomass was then placed into a large plastic syringe and manually squeezed through a muslin filter. Whilst acceptable for the preparation of samples for extraction profiling and artemisinin recovery in the laboratory, this system clearly could not begin to approximate the pressures or solvent removal efficiencies routinely used on an industrial scale and solvent losses per cycle were extremely large; simple weighing of the biomass before and after extraction evidenced a substantial increase in mass due to retained solvent, which was confirmed by ion chromatography to represent a typical loss of 15-20% of the initial mass of solvent per extraction cycle. Such a figure is obviously absurd in the context of a scaled-up industrial process and, in the absence of any means to readily simulate such conditions given the available equipment, this part of the study was not pursued further. The engineering issues of effective

solvent recovery from processed biomass therefore require addressing prior to the point of scale-up of any ionic liquid based artemisinin extraction method.

In view of the likelihood that, regardless of the efficacy of any recycling system, contamination of exhausted biomass with low levels of solvent is very likely, the possible effects of the IL entering the environment via this route should be considered. Whilst a detailed analysis is well beyond the scope of this study, it can be stated in summary that we have undertaken additional independent studies into the safety, biodegradability and both acute and environmental toxicity of DMEA oct and related ionic liquids. This work is ongoing; however it can be confirmed that DMEA and similar cations are readily biodegradable according to the ICH definition, being fully mineralized within 48 hours. When introduced into an aqueous microflora community, DMEA at concentrations up to 50mM was non-toxic and in fact served as a nutrient, eliciting a 3-log increase in the total viable cell count. A number of DMEA based ionic liquids have been screened for possible mutagenic or carcinogenic effects using the Mini Ames test, with negative results. In addition, once exposed to an aqueous environment, solvents such as DMEA oct dissociate into their individual component ions and the corresponding acid and base (in this case, octanoic acid and N,N-dimethylethanolamine); the toxicity and environmental effects of these components have been thoroughly evaluated by numerous independent sources and are publicly available.[12] Obviously, the specific health and safety issues associated with the use of any new solvent in any process would require downstream consideration prior to implementation.

4.5 Modelling of effect of impurities upon precipitation

Previous molecular simulation studies had involved modelling the interactions between ionic liquids, artemisinin and water in a 3-component system. Owing to the timings of the analytical work performed in the described study, simulation studies were initiated prior to confirmation of the identity of the major co-extracted impurities and had focussed on the terpenoids artemisinic acid and arteannuin B, which had been hypothesized as being likely to co-extract with artemisinin. As will already be apparent, however, these two compounds were not detected in the DMEA oct extract, hence modelling work involving these was terminated and a new series of simulation studies was initiated, aiming to evaluate the likely interactions between DMEA oct and casticin with and without the presence of water and artemisinin.

Initial simulations rapidly established the potential for the existence of numerous strong hydrogen bonding interactions between the hydroxyl groups of casticin and the corresponding functionalities of the DMEA cation, as well as with the ionized nitrogen. The output from the model suggested that the solubilization of casticin in DMEA oct was an extremely favourable process from a thermodynamic perspective and that the solubility of casticin in this solvent was likely to be considerably greater than that of artemisinin. Single solute molecular

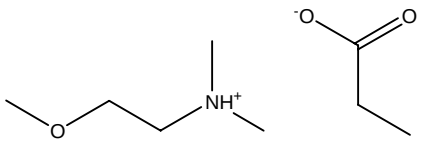
dynamic simulations predicted that each molecule of casticin would form a localized disruption in the ionic liquid lattice, in which the casticin was coordinated by at least four DMEA cations, with the octanoate anion side chains extending out into the ordered bulk solvent. The presence of significant levels of casticin would therefore be expected to markedly decrease the capacity of DMEA oct to dissolve high concentrations of artemisinin.

In the corresponding simulations representing the dissolution of artemisinin in DMEA oct,[2] the addition of water elicited a clear series of domain changes, through which the water permeated into and dissolved the ionic liquid, expelling the weakly-interacting artemisinin from solution. In the casticin simulation, however, a very different response was seen. Addition of one volume equivalent of water to the solvated casticin model resulted in the formation of a tightly hydrogen bonded lattice, in which water molecules interacted strongly with casticin and with DMEA cations, both in their own right and as bridging molecules. This simulation strongly suggested that the addition of water to the casticin/DMEA oct system would result in simple dilution, without any precipitation – an hypothesis borne out by experimental observation and evidenced by the lack of any casticin in the precipitated artemisinin fraction.

The high affinity of the extraction solvent and post-dilution supernatant for casticin do not necessarily suggest that the presence of this compound would adversely affect the precipitation of artemisinin following the addition of water – although this remains a possibility. Of more immediate concern was the clear implication that recovery or removal of casticin from the ionic liquid in order to recycle the solvent was liable to be extremely difficult if not impossible. Depending upon the actual concentration of casticin present – which could not be assayed due to lack of a pure standard – the continual accumulation of this compound (and the demethylated analogue) in the ionic liquid over a number of extraction cycles would be expected to exert an increasingly major effect upon the capacity of the ionic liquid to extract artemisinin.

4.6 Incremental improvement of ionic liquid structure

Rather than focus on a potentially fruitless effort to remove casticin from solution, the model was utilized to predict the effects of very basic changes to the structure of the ionic liquid upon the strength of solvent-solute interactions. Given that the major such interaction was predicted to involve hydrogen bonding between casticin and the DMEA cation hydroxyl groups, the initial single-parameter recommendation was to etherify the oxygen functionality to a methoxyl group. This modification was predicted to decrease the hydrophilicity of the ionic liquid to the extent that it may no longer fully dissolve into water in the precipitation step; hence in order to retain hydrophilicity without greatly increasing hydrogen bonding capability, the modelling studies suggested a reduction in the anion side chain length from C8 to C3. The incrementally improved ionic liquid structure thus suggested by the modelling studies was N,N-dimethyl(2-methoxyethyl)ammonium propionate (DMMOEA pro, **6**).



N,N-Dimethyl(2-methoxyethyl)ammonium propionate (DMMOEA pro, **6**)

Corresponding simulations to the above were run using casticin in DMMOEA pro. The most obvious difference between this and the preceding simulation was the relatively weak nature of hydrogen bond formation between the ionic liquid cation and the solute. The ether function serves solely as an hydrogen bond acceptor rather than as both acceptor and donor, hence the total number of interactions and the energies of individual interactions were both reduced. The dissolution of casticin in DMMOEA pro was thus anticipated to be significantly less favourable than in DMEA oct, and the disruption of the ordered ionic liquid structure was considerably less extensive.

4.7 Manufacture and analysis of improved ionic liquid

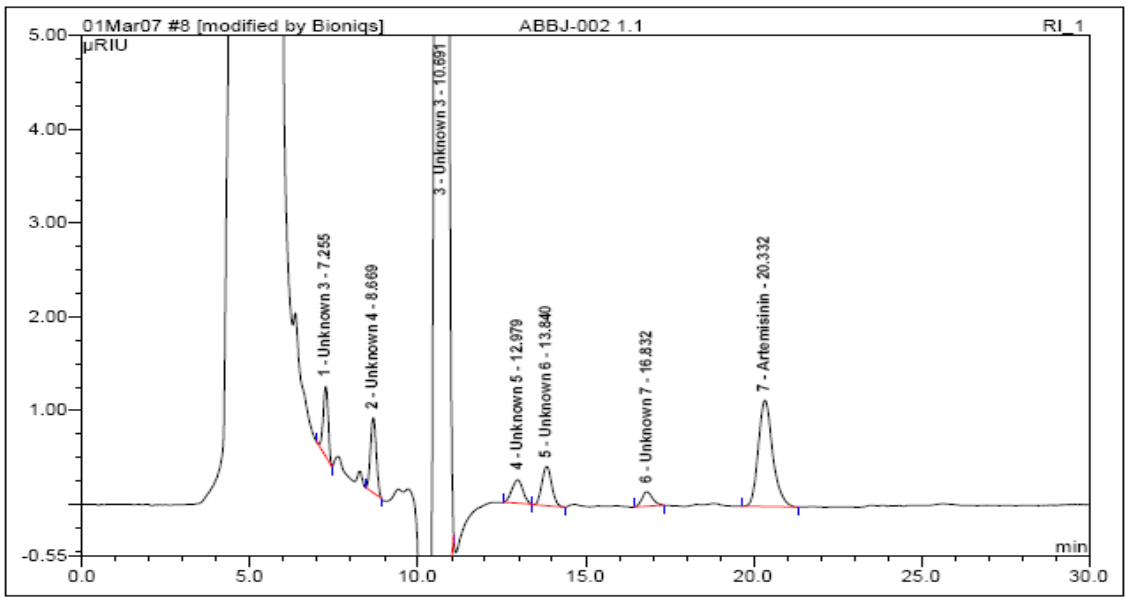
DMMOEA pro was manufactured by the direct neutralization of N,N-dimethyl-(2-methoxyethyl)amine with an equimolar amount of propionic acid, according to standard Bioniqs internal protocols. The precursor amine was not available commercially and had to be custom manufactured for the project. Following manufacture the ionic liquid was assayed for purity and water content as described above.

4.8 Extraction of artemisinin in improved ionic liquid

Extractions of *A. annua* were performed using DMMOEA pro, under identical conditions to those used with DMEA oct. Primary extracts were analysed by HPLC according to the standard protocol.

DMMOEA pro reproducibly extracted artemisinin to a level equivalent to 0.60 (+/-0.05)% by weight of the dried plant; a significant improvement over DMEA oct under identical conditions. The extraction profile (**Figure 13**) was substantially different to that observed with DMEA oct and was notable for the effective absence of the flavonoid components; however, a number of further unknown impurities were extracted. The retention times and RI responses suggested that these compounds may be terpenoid in nature, but in the absence of suitable standards and in view of time constraints these impurities were not identified. More significantly, the new ionic liquid proved difficult to elute sharply from the HPLC column under the standard conditions, resulting in a broad solvent peak effectively swamping all signals between 4-10 minutes, followed by a large secondary peak eluting after 10.7 minutes. Analysis of the neat ionic liquid confirmed the presence of these peaks, despite NMR and ion chromatography

indicating that the ionic liquid contained no measurable impurities. It may therefore be the case that an alternative column or eluent needs to be employed in order to effectively quantify the extraction profile of DMMOEA pro applied to *A. annua*.



No.	Ret.Time min	Peak Name	Height µRIU	Area µRIU*min	Rel.Area(ident.) %	Amount	Type
1	7.26	Unknown 3	0.746	0.129	0.68	0.366	BMB*
2	8.67	Unknown 4	0.800	0.162	0.85	0.459	BMB*
3	10.69	Unknown 3	64.690	17.977	94.01	50.950	BMB*
4	12.98	Unknown 5	0.250	0.093	0.49	0.263	BMB*
5	13.84	Unknown 6	0.418	0.142	0.74	0.404	BMB*
6	16.83	Unknown 7	0.155	0.056	0.29	0.158	BMB*
7	20.33	Artemisinin	1.133	0.564	2.95	1.599	BMB
Total:			68.191	19.123	100.00	54.198	

Figure 13. HPLC-RI chromatogram of primary extract of *A. annua* using N,N-dimethyl(2-methoxyethyl)ammonium propionate.

4.9 Recovery of artemisinin from improved ionic liquid

The method for the recovery of artemisinin from DMMOEA pro solution was at this stage restricted to the aqueous precipitation method previously employed. Experiments with pure artemisinin evidenced that the addition of 2 volumes of water to a solution of 50mg/mL artemisinin in DMMOEA pro reproducibly precipitated 85-88% of the dissolved artemisinin in the form of a crystalline white powder, with no detectable carry-over of solvent. In order to minimize the energy input involved in removing the water and recycling the ionic liquid, the relative volume of water added was restricted to between 0.5-2 equivalents of the volume of extract. This again proved sufficient to precipitate the bulk of the extracted artemisinin from solution (Table 2).

Volumes H_2O added	% artemisinin recovered	g IL per g artemisinin
0.5	85 +/- 10	67-89
1	88 +/- 5	53-80
1.5	82 +/- 8	27-38
2	88 +/- 6	13-20

Table 2. Recovery of artemisinin from DMMOEA pro solution.

Artemisinin was again recovered by means of filtration through a silica bed over a sinter. Although this was efficient in terms of recovering the precipitate, the same problem of ionic liquid contamination was observed here as it had been with DMEA oct. Furthermore, two of the unknown co-extractants also precipitated along with artemisinin, giving purity values for the recovered material of 80-87% excluding ionic liquid.

4.10 Microscope study of effect of solvent exposure upon trichomes

The effects of DMEA oct upon the trichome cells of *A. annua* were qualitatively compared with those elicited by chloroform, by optical observation of leaf surfaces at 64x magnification under a microscope during and immediately following the addition of a drop of the solvent.[9]

When exposed to chloroform, trichomes were observed to undergo rapid, vigorous and complete lysis in the space of a few seconds. By contrast, trichomes exposed to the ionic liquid underwent no apparent morphological changes, suggesting that the two solvents may effect the extraction of artemisinin by distinct and unrelated mechanism – rupture of the entire cell in the case of chloroform, compared with a more gentle permeation on the part of the ionic liquid. These experiments cannot be regarded as more than a superficial indication of the effects of the solvents upon trichomes and more detailed studies may be beneficial; however, the much cleaner extraction profile of DMEA oct compared to chloroform may well be at least in part attributable to the less destructive action of the ionic liquid on trichome cells.

4.11 Investigation of extraction technique

The original intention of this phase of the study had been to evaluate the effect of pre-milling the plant material to varying extents upon the extraction efficiency. However, due to the fact that the raw material supplied was already coarsely milled, it was impossible to perform a direct comparison with dry unprocessed leaf. Studies were therefore restricted to comparing the rate and yield of artemisinin extraction under the normal conditions described above with those obtained under more vigorous conditions (continuous grinding with mortar and pestle for the duration of the extraction process). Solvent:leaf ratio was 3:1 w/w. Time-course samples were taken and analysed by HPLC. On average, the more vigorous method resulted in a 10-15% increase in the level of artemisinin in

solution after 10 minutes but had minimal effect on the net total value extracted (Table 3).

Solvent	Extraction method	Artemisinin conc 10 mins mg/mL	Artemisinin conc 30 mins mg/mL
DMEA oct	Stirring	1.51 +/- 0.05	1.76 +/- 0.05
DMEA oct	Grinding	1.68 +/- 0.05	1.80 +/- 0.05
DMMOEA pro	Stirring	1.77 +/- 0.05	2.03 +/- 0.05
DMMOEA pro	Grinding	2.02 +/- 0.05	2.09 +/- 0.05

Table 3. Effect of grinding on rate and yield of artemisinin extraction in ionic liquids.

5. Discussion and recommendations

The work performed during the course of this study has yielded valuable information regarding the parameters necessary for the design of an ionic liquid for the extraction of artemisinin. The initial ionic liquid identified as a suitable candidate for basic testing, DMEA oct (**2**), has been exhaustively studied and has been shown to possess a number of desirable properties, notably its ability to extract artemisinin in the absence of large amounts of co-extracted terpenoids. However, a number of potential operational problems with this solvent have been identified, in particular its tendency to co-extract flavonoids (notably casticin) and the poor reproducibility of the precipitation from solution of artemisinin by the addition of water. DMEA oct is therefore not considered suitable for in-field application.

The utility and validity of the concept of utilizing molecular modelling to iteratively improve the performance of an ionic liquid in a given application has been demonstrated, through the simple example of incrementally modifying DMEA oct to give DMMOEA pro. This latter ionic liquid was specifically developed to avoid the co-extraction of flavonoids whilst remaining effective in isolating artemisinin, in which respects it was successful. It must be emphasized, however, that DMMOEA pro is not a final, optimized product ready for in-field deployment, owing to the co-extraction of low levels of terpenoids and the potentially high cost of sourcing a non-standard precursor amine. DMMOEA pro rather represents a stage further along the path of refining and developing an optimized ionic liquid; it constitutes an advance over DMEA oct but is still some way from commercial applicability. A comparison of the key performance aspects of DMEA oct and DMMOEA pro is shown in Table 4 below.

Solvent	Abs. saturation concentration of artemisinin (g/L) @25°C	Optimal extraction time (mins)	Primary yield vs chloroform	Precipitation yield	Purity after first recrystallization
DMEA oct	110	30-60	32%	82%	>97%
DMMOEA pro	68	10-45	41%	88%	>97%

Table 3. Comparative performance of DMEA oct and DMMOEA pro for artemisinin extraction

The hitherto speculative concept, that modifying the structure of an ionic liquid in a rational manner would dramatically influence its performance according to a stated series of objectives has however now been demonstrated. As a result, further fine tuning can lead to the end product of an ionic liquid optimized for the needs of the real process – namely, a solvent which is:-

1.) selective for artemisinin in the presence of flavonoids and terpenoids
2.) biodegradable and non-toxic
3.) capable of being sourced and recycled within the economic constraints of the overall operation of the process.

We therefore recommend that these parameters are used as the basis for a final product specification and that multi-parameter screening is used in conjunction with molecular modelling to produce this optimized solution.
In addition, we recommend the involvement of chemical engineering specialists in order to address process issues, such as more effective recovery of solvent from post-extraction biomass, recovery of precipitated artemisinin (and removal of residual ionic liquid) and removal of water from the post-precipitation supernatant in order to recycle the ionic liquid. Addressed together, optimization of both the solvent and the process is anticipated to deliver an efficacious and practical solution for the extraction of artemisinin.

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