Molecular design of polymers for artemisinin

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Medicines for Malaria Venture (MMV)

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The main objective of the proposal was the development of polymeric material capable of the selected sequestering of the artemisinin (a drug used to treat multidrug resistant strains of Falciparum malaria).

The project was divided in three parts:

1. The computational design of polymer with specificity for artemisinin
2. Polymer synthesis and testing
3. Evaluation of artemisinin purification from the plant extracts

Molecular design of polymers for artemisinin

The rational design of affinity polymers was carried out on a Silicon Graphics Octane workstation running the IRIX 6.5 operating system. The workstation was configured with two 195 MHz reduced instruction set processors, 1 Gb memory and a 20 Gb fixed drive. The system was used to execute the software packages SYBYL 7.0 (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA).
The rational design protocol involves 3 steps:

1. Design of functional monomer database;
2. Design of molecular model of template to be screened;
3. Screening using a LEAPFROG^{TM} algorithm.

Design of Functional Monomer Database

The first step involved the design of a virtual library of functional monomers. These monomers possess polymerisable residues and residues capable of interacting with a template through electrostatic, hydrophobic van der Waals forces, and dipole-dipole interactions. Preferred monomers were those that are able to interact with the template through non-covalent interactions and that can be polymerised through a radical mechanism.

The library designed for this project contained 22 of the most commonly used functional monomers, these being acidic, basic or neutral molecules. Most of the selected monomers were described extensively in the literature as a result of their ease of polymerisation by temperature conditions or photo-initiation, availability and cost.

The charges for each atom of each monomer were calculated and the structures of the monomers refined using molecular mechanical methods. All the monomers in the database are energy minimised individually to a value of 0.001 kcal mol^{-1}. The structures of the monomers in the database are shown in Figure 1.

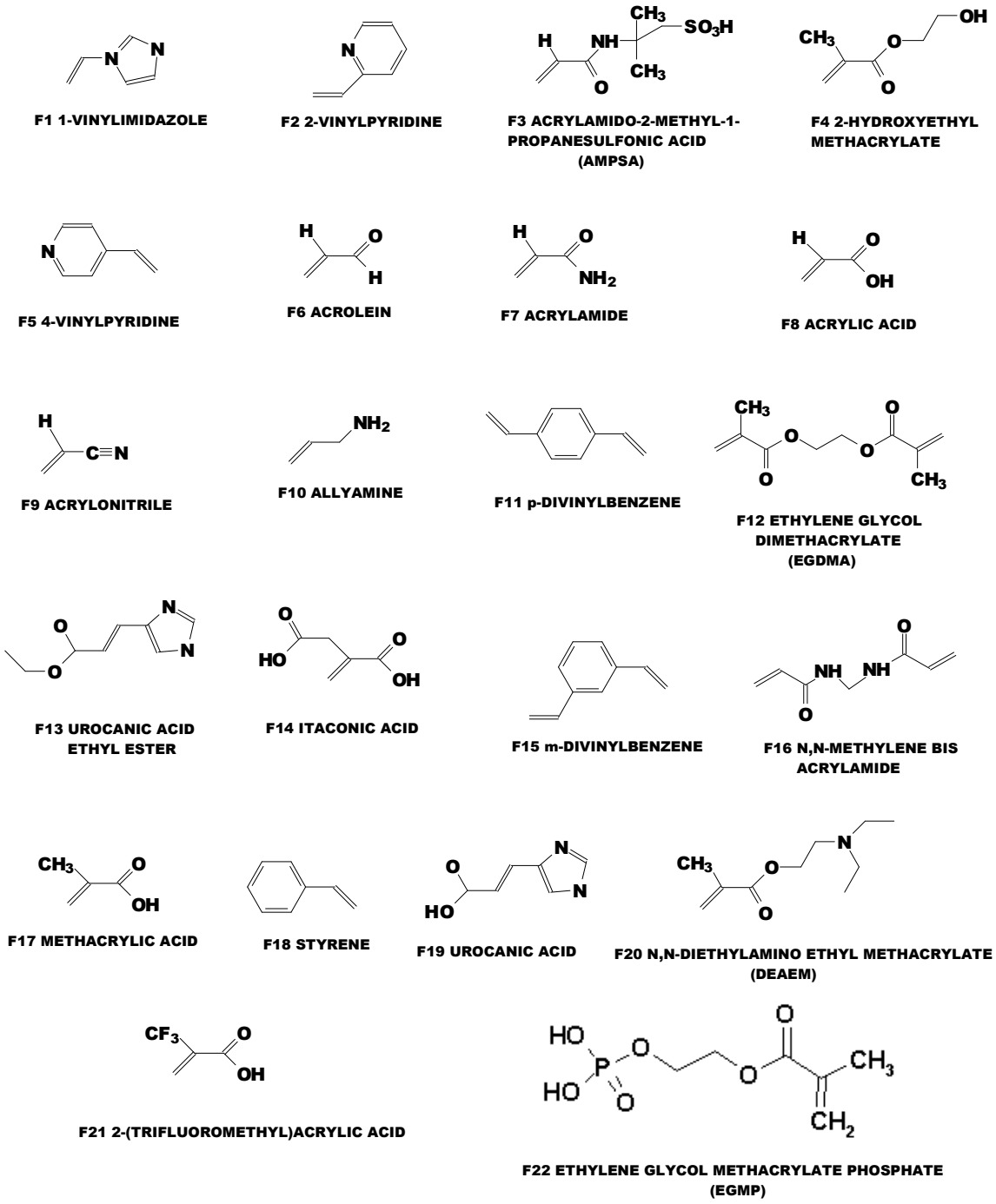

Figure 1. Virtual library of functional monomers.

Design of Molecular Model of Template

For the second step, the template was modelled in a similar manner to that of the monomers. The charges for each atom on the template were calculated and the structures refined using molecular mechanical methods. Energy minimisation was performed to a value of 0.001 kcal mol^{-1}. The template artemisinin in its neutral form was minimised using a dielectric constant of 1.

A minimised structure of artemisinin is shown below (Figure 2). The oxygen atoms are shown in red and the nitrogen atoms are shown in dark blue. The white atoms are carbon and the light blue atoms are hydrogens. This structure was charged with the Gasteiger-Huckel approximation method, and refined by the molecular mechanics method applying an energy minimisation using the MAXIMIN2 command.

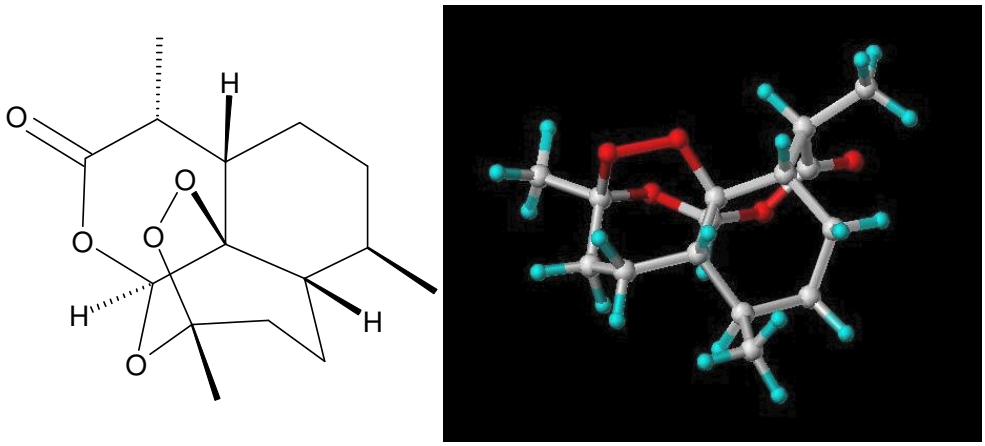

Figure 2. Structure of artemisinin (left) and minimised structure of artemisinin (right).

Screening

Each of the monomers in the database was then probed for its possible interaction with the template (artemisinin). The LEAPFROG^{TM} algorithm was used to screen the library of functional monomers for their possible interactions with the template. The program was applied for 40,000 iterations. The results from this were examined and the empirical binding score evaluated. Monomers giving the highest binding scores represented the best candidates for polymer preparation and for forming the strongest complexes with the template.

The template (artemisinin) was screened against the charged and neutral forms of the monomers. The monomers were charged depending on whether nitrogen or a carboxylic acid group was present in order to reflect possible scenario during the polymerisation or rebinding conditions(charged and neutral forms of the monomers are denoted by a + or – sign). These simulations reflect the ability of the templates to bind to charged and neutral monomers in the modelling experiments. Based on Cranfield's previous experience, it is expected that under laboratory conditions binding and recovery will require either basic and/or acidic conditions. This would undoubtedly mean that the monomers may be charged in some form, and hence these effects needed to be reflected in the computational modelling experiments. Using the LEAPFROG™ algorithm for the screening of artemisinin resulted in a table ranking the monomers with the highest binding score (kcal mol^{-1}), as the best candidates for polymer preparation, was generated.

For artemisinin, the top five highest binding monomers were identified as N,N-(diethylamino)ethyl methacrylate (DEAEM), N,N-methylenebisacrylamide (MBAA), acrylamide, acrylamido-2-methyl-1-propanesulfonic acid (AMPSA) and 2-(trifluoromethyl)acrylic acid (TFMAA) as shown in Table 1.

Table 1. Binding energy between artemisinin and selected functional monomers.

Rank	Monomer	Binding Energy (kcal mol^{-1})
1	+DEAEM	-30.78
2	MBAA	-27.90
3	Acrylamide	-25.73
4	AMPSA	-20.53
5	TFMAA	-17.50

+ denotes protonated form

The monomer-template molecular complexes are shown in Figures 3-7. The dotted lines- are hydrogen bonds which show the interactions between functional groups of artemisinin and functional monomers.

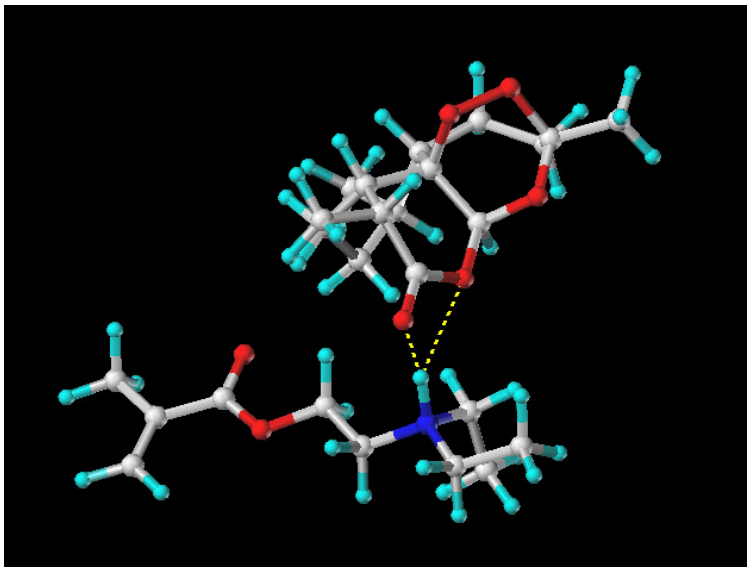

Figure 3. Artemisinin- +DEAEM molecular complex.

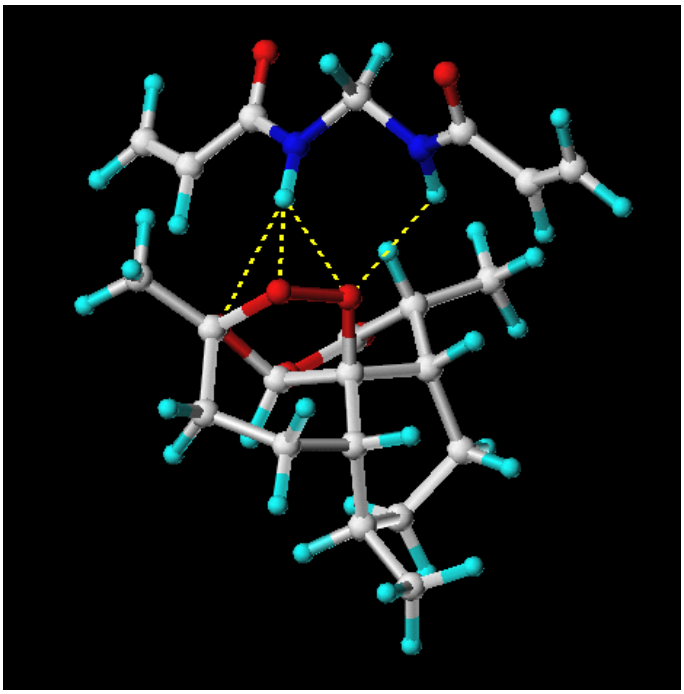

Figure 4. Artemisinin- MBAA molecular complex.

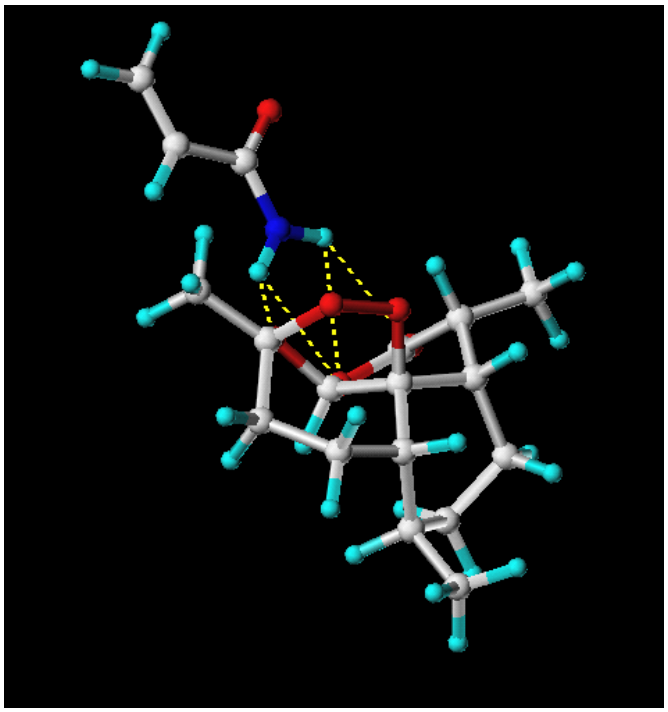

Figure 5. Artemisinin- acrylamide molecular complex.

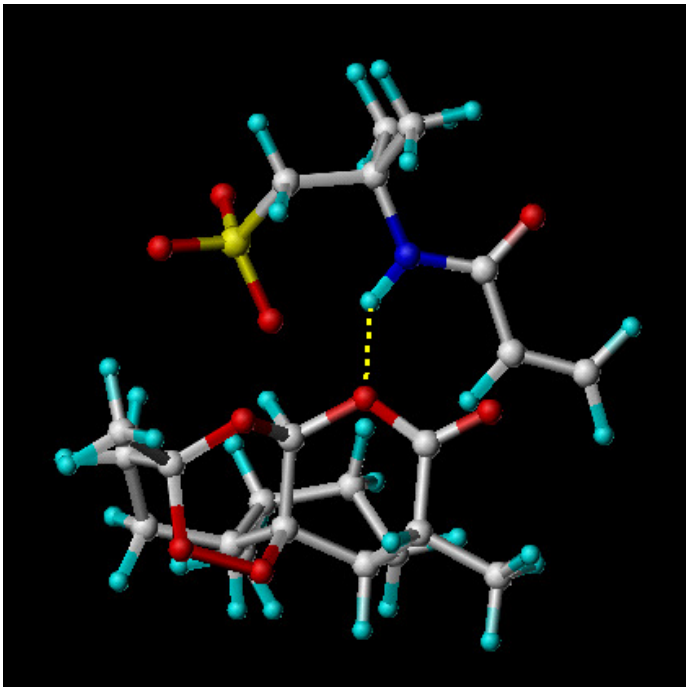

Figure 6. Artemisinin- AMPSA molecular complex.

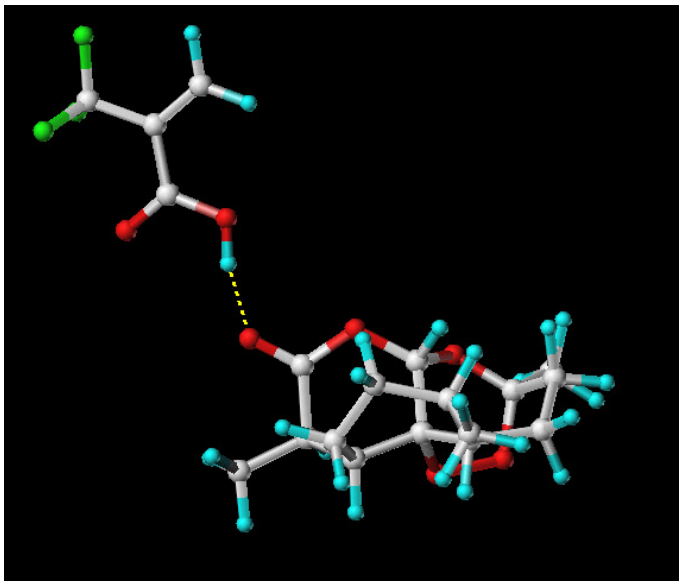

Figure 7. Artemisinin- TFMAA molecular complex.

Polymer synthesis and testing

Four functional monomers were used for the polymer preparation based on their strength of binding to artemisinin. Among these monomers were N, N-(diethylamino) ethyl methacrylate (DEAEM), N, N-methylenebisacrylamide (MBAA), acrylamide-2-methyl-1-propanesulfonic acid (AMPSA) and 2-(trifluoromethyl)acrylic acid (TFMAA).

The composition of polymers was as follows: 5 g of functional monomer, 20 g of cross-linker (ethyleneglycol dimethacrylate, EGDMA), 25 g of porogen (dimethylformamide, DMF), 500 mg of initiator (1,1-azobis (cyclohexanecarbonitrile)). The polymers were prepared by temperature-induced polymerisation in the oil bath at 80 ºC for 12 hours. The resulted bulk polymers were ground and sieved in methanol. The fraction with particle size in the range from 25 to 106 µm was collected and dried. The empty 1-mL cartridges (Supelco, UK) were packed with 100 mg of polymer and tested using HPLC-MS (Waters, UK).

Quantification of artemisinin using HPLC-MS

The quantification of artemisinin was performed using Waters HPLC in tandem with a bench-top triple quadrupole mass spectrometer model Micromass Quatro Micro (Waters, UK) equipped with an electrospray probe. The values of the voltages applied to the sampling cone (40 V), capillary (3.5 V) and extractor (1 V) were optimised by continuous infusion in order to achieve the highest possible sensitivity for artemisinin. The electrospray probe was maintained at +350 °C with a spray voltage of 450 V in the positive ionization mode. The electron multiplier was set at 650 V. HPLC-MS analyses were carried out in Selected Ion Recording mode (SIR), where two masses (*m/z* 209 and *m/z* 305) were monitored (Figure 8).

HPLC conditions were following: mobile phase A- 1 mM ammonia acetate buffer adjusted to pH 5.0, mobile phase B- methanol, flow rate- 0.2 ml min^{-1}, column temperature- 40 °C, column- Luna 3 µm, i.d.- 3 mm, length- 50 mm (Phenomenex, UK). The initial composition of 50:50 was maintained for 1 min; next the methanol content was increased linearly to 80% over a period of 6 min and maintained for 18 min. Re-equilibration time was 5 min between the runs. The samples injection volume was 10 µl for all samples. The quantification was performed using MassLynx software and peak of artemisinin with t_R= 9.9 min was quantified in total ion count (TIC) mode (Figure 9).

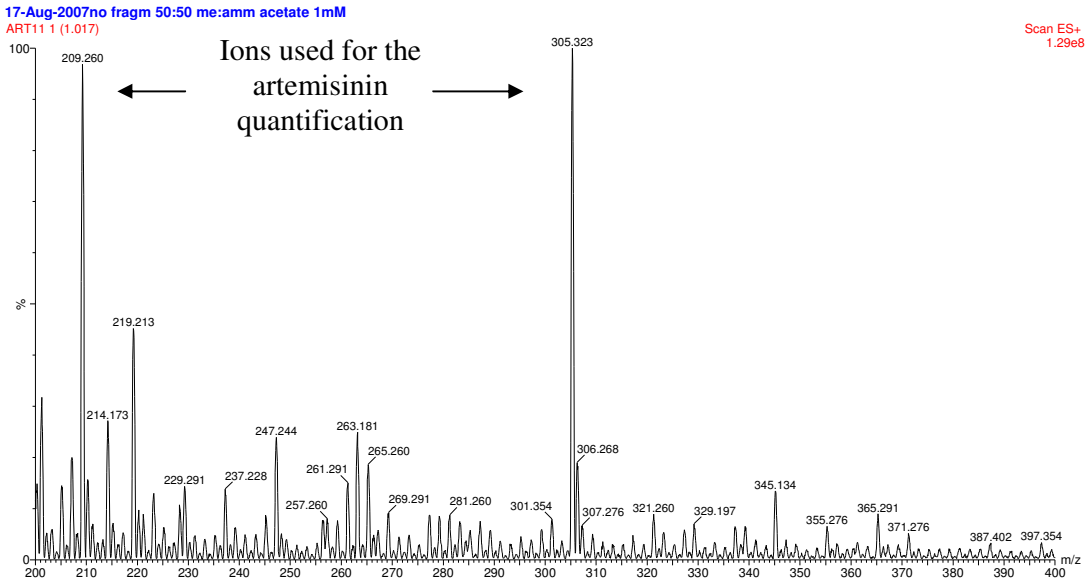

Figure 8. ESI mass spectrum of artemisinin in continuum mode.

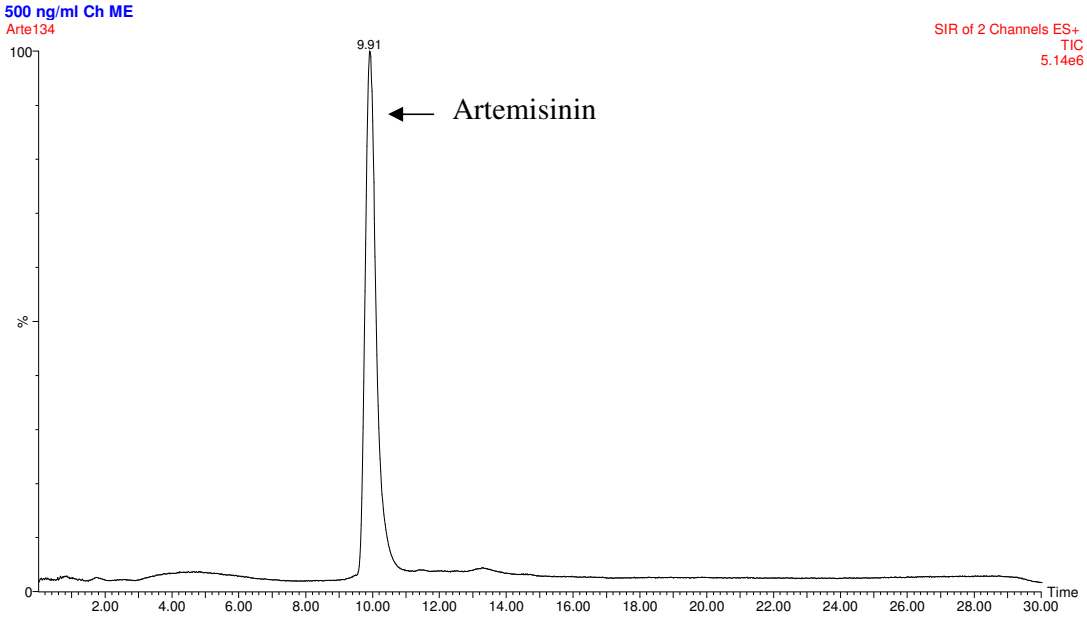

Figure 9. Typical HPLC-MS chromatogram which shows the peak of artemisinin (500 ng mL^{-1}).

The HPLC-MS method was very sensitive and reproducible and allowed the artemisinin quantification over the broad concentrations range (Figure 10).

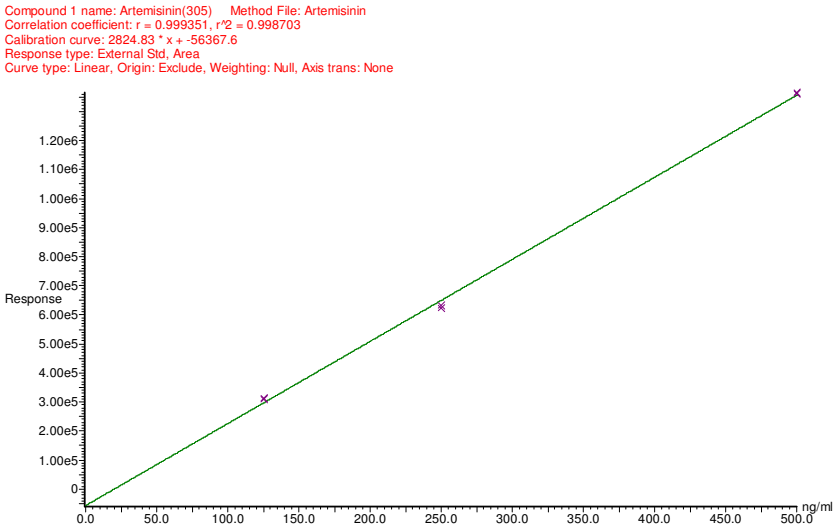

Figure 10. Typical calibration curve for quantification of artemisinin (correlation range- 0.999).

Polymers screening

Four prepared polymers (TFMAA-, MBAA-, DEAEM- and AMPSA-based) were packed into 1-mL SPE tubes and conditioned with 1 mL of hexane on the vacuum

manifold (Supelco, UK). 1 mL of the artemisinin solution in hexane (500 ng mL^{-1}) was loaded on the cartridges. The hexane solution passed through the cartridge was collected in the glass vials. The collected hexane samples were dried out using a TurboVap evaporator under the stream of nitrogen at 35 °C and were reconstituted in the methanol for quantification. It was found that MBAA-based polymer demonstrated the best binding of artemisinin from hexane (85%), followed by TFMAA (83%), DEAEM (79%) and AMPSA (76%). The MBAA-based polymer was selected for the optimisation of SPE method.

Solid phase extraction

Hexane was selected as the loading solvent based on the method of extraction of artemisinin from the plants which was published in the literature (P. Sahai, R. Vishwakarma, S. Bharel, A. Gulati, A. Z. Abdin, P. S. Srivastava, S. K. Jain HPLC-electrospray ionization mass spectrometric analysis of antimalarian drug artemisinin. Anal. Chem., 70 (1998) 3084-3087).

SPE protocol:

1. Conditioning of the cartridge using 1 mL of hexane;
2. Loading: 1 mL of hexane spiked with artemisinin. For the best adsorption it was recommended to control the flow rate. The suitable flow rate is 0.5 ml min^{-1};
3. Washing with 2 mL of hexane followed by drying the SPE tube in the vacuum manifold for 1.5 min in order to evaporate the remaining hexane;
4. Elution of artemisinin with methanol (2x 1 mL).

Polymer capacity measurement

In order to measure the polymer capacity towards the artemisinin the breakthrough curve was obtained. Artemisinin solution with concentration 1 mg mL^{-1} in hexane was loaded onto the SPE cartridges. The artemisinin concentration in the filtrate was measured using a spectrophotometer (Hitachi, Japan) at λ=195 nm and was quantified using the calibration curve which is shown on Figure 11.

The breakthrough volume was considered as the volume from which more than 50% of artemisinin was adsorbed.

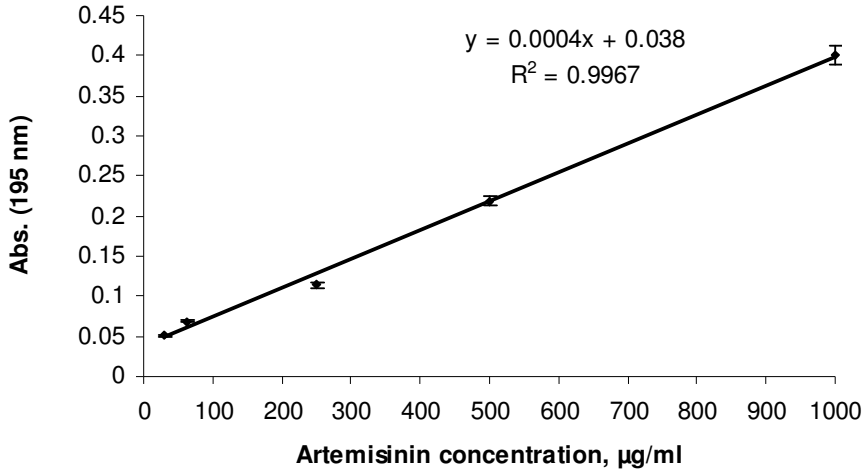

Figure 11. Calibration curve for quantification of artemisinin in hexane.

It was found that it is possible to adsorb more than 50% of artemisinin from 15 mL of hexane solution with concentration 1 mg mL^{-1}. The amount of adsorbed artemisinin was estimated as 12 mg per 100 mg of the polymer (120 mg per gram of the polymer) and the bed volume (the ratio between volume of crude extract from which ≥ 50% could be adsorbed and volume of resin) was estimated as 150 (Table 2).

Table 2. The capacity of MBAA-based polymer towards the artemisinin during the extraction from 1 mg mL^{-1} solution in hexane. The SPE cartridges are packed with 100 mg of the polymer.

Volume of loaded solution	Amount adsorbed, mg	Adsorption, %
1	0.870	87
2	0.845	84.5
3	0.882	88.25
4	0.960	96
5	0.910	91
6	0.835	83.5
7	0.812	81.25
8	0.697	69.75
9	0.785	78.5
10	0.802	80.25

11	0.747	74.75
12	0.822	82.25
13	0.745	74.5
14	0.697	69.75
15	0.630	63
Total adsorbed, mg	**12.04**	
Total adsorbed, %	**80**	
Effective bed volume*	**150**	

* Ratio between volume of extract from which ≥ 50% could be adsorbed and volume of polymer

Purification of artemisinin from plant extracts using MBAA-based polymer

The goal of this part of the project was to determine if selected MBAA- based polymer would be able to extract the artemisinin from hexane plant extract.

Tasks:

1. Evaluate how much artemisinin is adsorbed by polymer from hexane extract;
2. Calculate the polymer capacity;
3. Optimise and evaluate the elution of artemisinin;
4. Evaluate the possibility of polymer regeneration and re-use (stability).

In comparison with model sample of artemisinin where only very minor impurities were expected it was found that it is not possible to use the spectrophotometer for quantification of the extraction from plant extract due to presence of interfering compounds which also adsorb at λ= 195 nm. Therefore, all consequent quantification of artemisinin was conducted using HPLC-MS following the method described earlier.

The starting point of purification was decided to select the stage where 266,000 L of hexane were used to extract the 13,288 kg leaves. Calculations are based on the information that artemisinin content in leaves is 0.51% and artemisinin left in spent leaves- 0.16% therefore it is expected that 0.35% of the weight of the leaves is artemisinin (46.5 kg in 266,000L). It is understood that the final hexane concentrated

(paste) was prepared by concentration of original extract to 500 L (concentration factor- x532).

In order to prepare the "primary" plant extract 100 mg of the paste was dissolved in 53.2 mL of hexane using ultra-sonication for 30 min.

In order to measure the polymer capacity towards the artemisinin in plant extract the breakthrough curve was obtained. Hexane extract was loaded onto the SPE cartridges packed with 100 mg of MBAA-based polymer. The artemisinin concentration in the filtrate was measured using HPLC-MS and was quantified using the calibration curve which are shown on Figure 12. All samples for HPLC-MS quantification were diluted 200 times in acetonitrile. The artemisinin concentration in the "primary" hexane extract was quantified as 0.24 mg mL^{-1}.

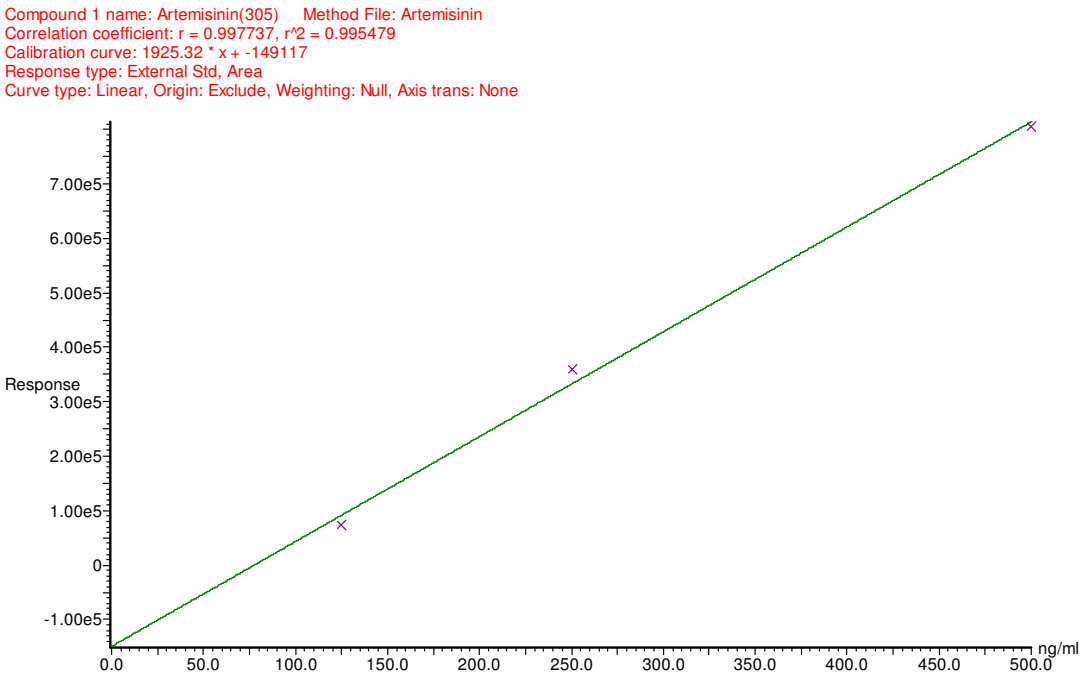

Figure 12. Calibration curve for artemisinin quantification using HPLC-MS.

Table 3. The capacity of MBAA-based polymer towards the artemisinin during the extraction from the "primary" hexane extract. The SPE cartridges are packed with 100 mg of the polymer.

Volume of loaded solution	Amount adsorbed, mg	Adsorption, %
1	0.214	90
2	0.224	94
3	0.231	97
4	0.234	98
5	0.235	99
6	0.235	99

7	0.234	99
8	0.234	99
9	0.233	98
10	0.230	97
11	0.221	93
12	0.206	87
13	0.179	75
Total adsorbed, mg	**2.9**	
Total adsorbed, %	**94**	
Effective bed volume	**130**	

Based on the experiments with "primary" hexane extract the capacity of the polymer was quantified as 29 mg per g of the polymer. It was interesting to evaluate if the increase in the concentration of the hexane extract and, correspondingly, in the concentration of artemisinin in the loading solution would result in a higher capacity of the polymer. Accordingly to the report prepared for MMV by M. Cutler, A. Lapkin and P. Plucinski ("Comparative assessment of technologies for extraction of artemisinin", p. 17) the artemisinin has limited solubility in hexane (0.46 g L^{-1} at 40 ºC). In accordance with artemisinin solubility "double hexane extract" was prepared: 200 mg of the concentrated paste was dissolved in the 53.2 ml of hexane under the ultra-sonication for 30 min and was filtered through 0.22 µm nylon syringe filter (Phenomenex, UK). This extract was considered as the most concentrated artemisinin solution in hexane which would allow performing the SPE without the risk of the artemisinin precipitating in solution. The polymer capacity for the "double hexane extract" was estimated as 60-70 mg of artemisinin per 1 g of the polymer.

As it was already mentioned that spectrophotometer could not be used for quantification of artemisinin in the hexane extract due to interference with other compounds and "invisibility" of artemisinin in the UV due to lack of chromophores. Nevertheless, the spectrophotometer could give the illustration how much of these interfering compounds were removed during SPE while the artemisinin was adsorbed on the resin (Figure 13). It is possible to see that the major part of the absorbance peak at λ= 195 nm originates from interfering compounds (compare the spectrum of 0.2 mg mL^{-1} of artemisinin in methanol and spectrum of un-purified extract, Figure 13). Since the results of the HPLC-MS quantification clearly show that artemisinin was adsorbed quantitatively from 1-13 mL of hexane extract (Table 3), and it is also clear that more than 50% of impurities have not been retained on the polymer during the loading step.

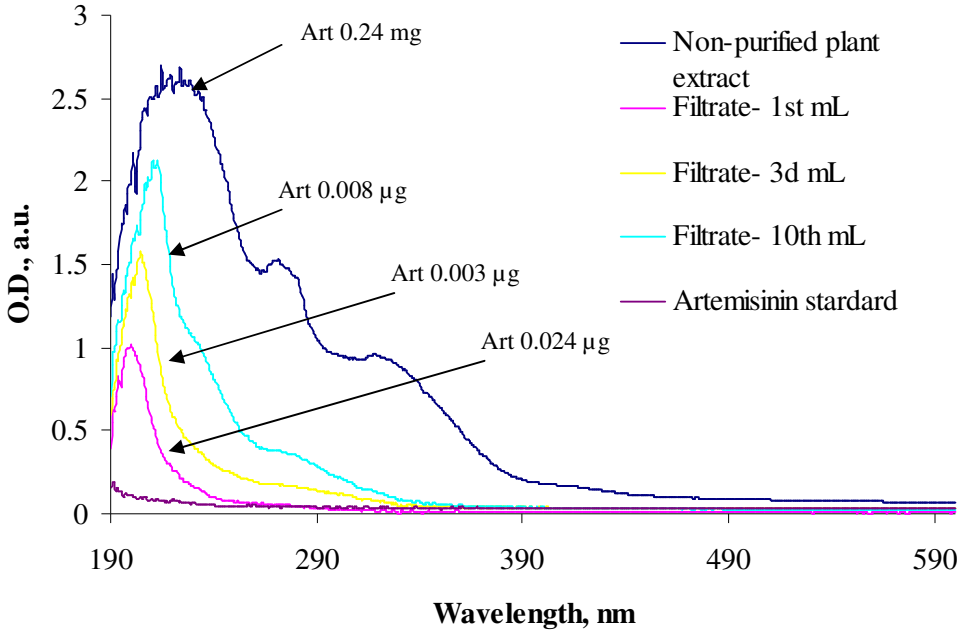

Figure 13. Purification of artemisinin from plant extract during the loading. Absorbance of the filtrates was measured using a spectrophotometer. The amount of artemisinin in the corresponding fractions was quantified using HPLC-MS. For the reference the spectrum of artemisinin standard with concentration 0.2 mg mL^{-1} is shown.

Elution optimisation

Elution with acetonitrile. 10 mL of hexane extract (the loaded extract was quantified as containing 0.292 mg mL^{-1} of artemisinin) were filtered through the SPE cartridge packed with 100 mg of the MBAA-based polymer. 3 x 1 mL of acetonitrile was used to elute the adsorbed artemisinin. The artemisinin concentration in each millilitre was quantified separately. It was found that most of the sample was eluted with 1st mL of eluent (Table 4).

Table 4. Elution of artemisinin using acetonitrile.

Volume, mL	**Concentration of artemisinin, mg mL^{-1}**	**Recovery, %**
Elution 1 (1st mL)	2.4	82
Elution 2 (2nd mL)	0.004	0.14
Elution 3 (3^{d} mL)	0.003	0.1

Samples subjected to SPE and eluted in acetonitrile were dried and weighed. The weight was compared with a dried original extract. It was found that the weight of non-purified extract was 24.2 mg and weight of the eluted fraction after SPE was 9 mg. This data suggested that quantitative recovery of the artemisinin (Table 4) and also almost 3 times purification were achieved.

Since the artemisinin was pre-concentrated approximately 10 times and it was eluted in the first millilitre of acetonitrile, it was possible to observe the crystallisation of artemisinin during the elution.

Elution with methanol. 10 mL of hexane extract (containing 0.32 mg mL^{-1} of artemisinin) were filtered through the SPE cartridge packed with 100 mg of the MBAA-based polymer. 3 x 1 mL of methanol was used to elute the adsorbed artemisinin. The artemisinin concentration in each millilitre was quantified separately. It was found that most of the artemisinin was eluted with 1st mL of methanol (Table 5). Methanol would be considered as preferred eluent for the practical application because it is cheaper than acetonitrile.

Table 5. Elution of artemisinin using methanol.

Volume, mL	**Concentration of artemisinin, mg mL^{-1}**	**Recovery, %**
Elution 1 (1st mL)	2.7	84
Elution 2 (2nd mL)	0.13	4
Elution 3 (3^{d} mL)	0.12	3.75

Samples subjected to SPE and eluted in methanol were dried and weighed. The weight of the eluted sample was compared with the dried original extract. It was found that the weight of non-purified extract was 25 mg and the weight of the eluted fraction after SPE was 15 mg. This data suggested that quantitative recovery of the artemisinin (84%) and approximately 2 times degree of purification were achieved.

Similarly to the previous experiment when acetonitrile was used as an eluent it was also possible to observe the crystallisation of artemisinin in the 1st ml of methanol during the elution.

Optimisation of regeneration

Several different solvents were tested in order to optimise the regeneration of MBAA-based polymer from the impurities of the plant extract. It included methanol, isopropyl alcohol, acetone, ethyl acetate and THF.

It was found that the best solvent for regeneration of the cartridges is ethyl acetate. The 100-mg cartridge used for the artemisinin purification from the plant extract was washed with 1 mL of ethyl acetate which removed all visible impurities which were retained on the cartridge. The regenerated cartridges were tested for adsorption of artemisinin and elution was quantified using HPLC-MS. It was found that the use of ethyl acetate for regeneration of the polymer does not affect loading and recovery of loaded artemisinin. Even following 10 regeneration cycles it was possible to recover 85-90% of the loaded artemisinin. The investigation of the numbers of allowed regeneration cycles was performed. Very similar results were obtained when THF was used for the regeneration. The important observation was that for successful regeneration it should be conducted immediately after the artemisinin elution. It allowed removing completely the adsorbed impurities. The complete regeneration of the polymer which was dried out after the elution was less successful.

In parallel, the testing has been performed to optimise the flow rate suitable for loading of artemisinin extract onto MBAA cartridges. Apparently the flow rate (in studied range) had no significant impact on polymer performance (Table 6).

Table 6. Dependence of the artemisinin recovery on flow rate.

Loading flow rate (controlled value), ml min^{-1}	**Recovery, %**
1.4	90±0.2
2	87.5±0.1
2.5	89.6±2.3

Further optimisation of elution

As it was shown earlier acetonitrile and methanol remove very effectively the artemisinin from the polymer. Unfortunately some impurities were also eluted together with artemisinin. In order to improve the sample purity additional optimisation of the elution solvents was conducted. The goal was to find the solvent or mixture of the solvents which would elute the artemisinin from the polymer but would not remove the impurities. Since the artemisinin and impurities are both soluble in hexane and therefore have quite similar physical properties the careful selection of the elution solvents was required. The selection was based on the value of *LogP* (partition coefficient) of the solvents. It was known that hexane does not remove both artemisinin and impurities from the polymer (*LogP* of hexane is 3.4). The idea was that some addition of the more polar solvents which are mixable with hexane could help to tune the elution profile and elute artemisinin with minimal impurities. Among suitable solvents were tetrahydrofuran (THF) and isopropyl ester (IPE).

For all following experiments 200 mg of concentrated paste was dissolved in 53 mL of hexane using ultra-sonication for 30 min and filtered through 0.2 µm nylon syringe filter (Phenomenex, UK). 100 mg of the MBAA-based polymer (25-106 µm) was packed in the 1-mL tubes (Supelco, Dorset, UK) and used for SPE experiments.

The SPE protocol was following:

1. Pre-conditioning: 2 mL of hexane;
2. Loading: 10 mL of hexane extract, flow rate- 2.5 mL min^{-1};
3. Washing: 2 mL hexane;
4. Elution: 3 mL of the eluent (hexane:50% IPE, cyclohexane:50% IPE, hexane:10%THF, hexane:15%THF and hexane:20%THF, (v/v));
5. Regeneration: 2 mL of ethyl acetate.

All elution samples were collected and characterised in respect to artemisinin concentration (quantified using HPLC-MS as it was described earlier) and to the level impurities (measured using spectrophotometer). In order to measure the UV-VIS spectra the samples were diluted in THF (1:10 for the loading sample and 1:33 for the eluted samples, proportionally to the degree of pre-concentration (x3.3)). Additionally,

all fractions collected during SPE were checked in relation to the content of artemisinin in the samples. For the HPLC-MS quantification of artemisinin all samples were diluted in methanol 1000 times and quantified using spiked standard solutions in methanol.

It was found that all tested eluents demonstrated a significant degree of purification in comparison with original extract (Figure 14). The purest samples were obtained when elution was conducted with hexane:50% IPE and hexane:10% THF.

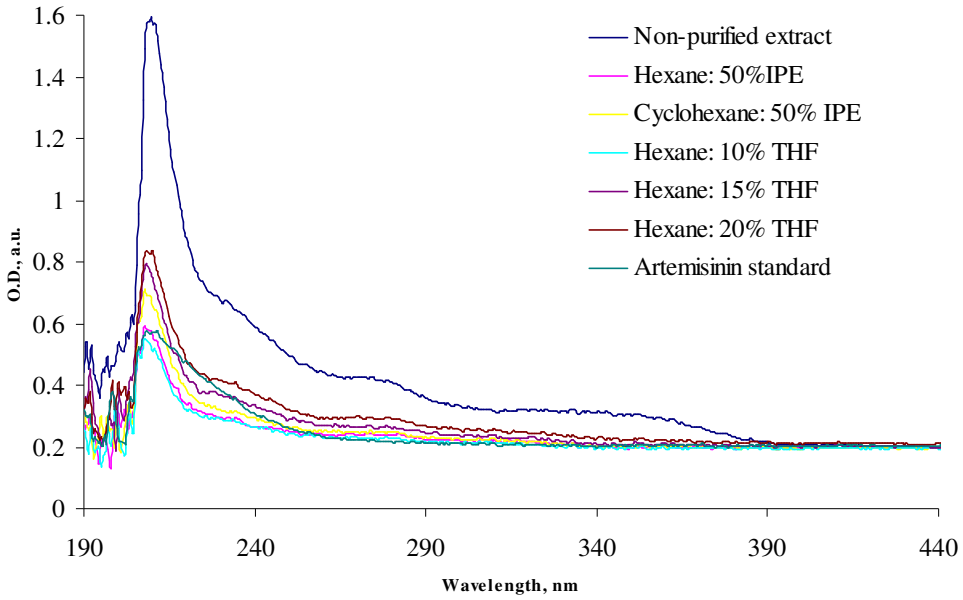

Figure 14. Spectra of eluted samples in comparison with non-purified extract and pure artemisinin (artemisinin standard- 2 mg mL^{-1} in THF).

In order to evaluate the elution in relation to effectiveness of artemisinin recovery the concentration of artemisinin in the eluents were quantified using HPLC-MS. It was found that although hexane:50% IPE and hexane:10% THF did not remove the impurities; they also did not elute artemisinin quantitatively (Table 7). Based on the degree of purification in combination with quantitative recovery of artemisinin, the hexane:15% of THF was considered as the best solution for the artemisinin elution (Table 7).

Table 7. Recovery of artemisinin from MBAA-based polymer using different eluents.

Eluents	**Purification degree (plant extract/eluted sample)**	**Artemisinin, mg**	**Recovery, %**
Loaded hexane extract	-	7.4	**-**
Hexane: 50% IPE	4	4.11	**55.6**
Cyclohexane: 50% IPE	3.4	5.04	**68**
Hexane: 10% THF	5.5	3.93	**53**
Hexane: 15% THF	2.5	6.65	**89.9**
Hexane: 20% THF	2.1	6.38	**86**

Quantification of artemisinin during the SPE steps

In order to demonstrate the distribution of artemisinin during the SPE steps all samples were quantified using HPLC-MS. The results of the quantification are shown in the Table 8. SPE of artemisinin from hexane extract was conducted following the SPE protocol described above. For the elution of artemisinin the hexane:50% IPE and hexane:15% THF were used as eluents.

Table 8. Quantification of artemisinin during SPE steps (hexane extract).

SPE steps	**Hexane: 50% IPE**		**Hexane: 15 % THF**	
	Art, mg	**Recovery, %**	**Art, mg**	**Recovery, %**
Hexane extract, 10 mL	7.88	-	6.5	-
Filtrate, 10 mL	0	0	0	0
Wash, 2 mL of hexane	0.13	1.6	0.1	1.5
Elution, 3 mL of eluent	3.5	44.4	6.46	99.3
Regeneration, 2 mL of ethyl acetate	3.8	48.22	0	0

It was found that hexane: 15 % THF removed completely all artemisinin adsorbed on the polymer and showed reasonable purification of the sample.

In order to estimate better the degree of purification the eluted samples were dried and weighed. 10 mL of the non-purified hexane extract and 3 mL of eluted samples were dried under nitrogen flow using TurboVap LV Concentration Workstation (Zymark, Hopkinton, MA, USA). It was found that original sample contained 46.4 mg of dry weight. The weight after elution was 11.8 mg for hexane:50% IPE eluent and 17.7 mg for hexane:15% THF eluent. The dry residue of non-purified extract contained 14% of artemisinin. Eluted sample using hexane:15% THF contained 55% of artemisinin. Thus, the polymer allowed achieving 4 times purification of the original sample.

For the illustration of the purification process the spectra of each fraction collected during SPE was recorded. It is possible to see the significant reduction of the optical density of the eluted samples and the amount of impurities retained on the polymer and removed during the regeneration (Figures 15, 16). It is also possible to see that the spectrophotometry could not be used for quantification of the artemisinin in the sample due to its low visibility and a big quantity of the interfering compounds which also adsorb in the range 190-215 nm. HPLC-MS could be considered as the most reliable quantification method which could be used for quantification of artemisinin in all fractions of the SPE.

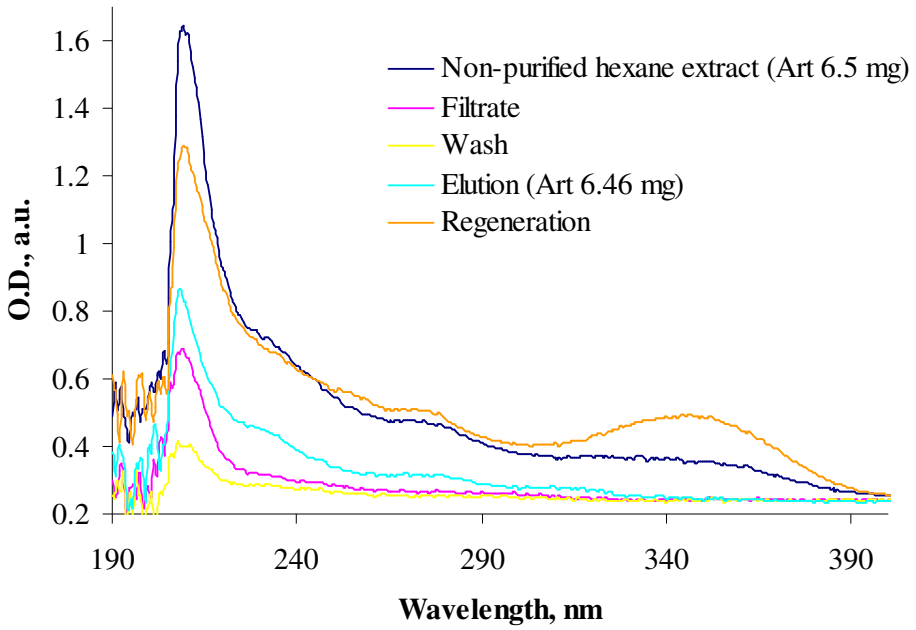

Figure 15. Typical spectra of the different fractions collected during SPE (the eluent-hexane: 15% THF). The artemisinin (Art) was quantified using HPLC-MS.

Figure 16. The picture of the fractions of the SPE.

Feasibility study of ethanol extract of artemisinin

(These experiments were performed outside of the scope of the project)

10 mL of ethanol extract were dried under vacuum using a rotor evaporator for 1 h, following by drying in the freeze-drier for 4 h. The dry pellet was re-suspended in the 10 mL of hexane using ultra-sonication for 30 min. The reconstituted sample was filtered through 0.2 µm syringe filter. 1 mL of the reconstituted in hexane ethanol extract (in the future referred as "ethanol extract") was loaded onto the 100-mg MBAA-based polymer packed in the 1-mL SPE tubes. The elution was performed using 1 mL of methanol. The artemisinin concentration was measured in all samples using HPLC-MS upon 1000 dilution in methanol. In order to measure the UV-VIS spectra, all samples were diluted 10 times in THF. Ethanol extract appeared substantially purified by SPE (Figure 17) and the polymer capacity was similar to the capacity in hexane. After the solid-phase extraction of the ethanol extract it was possible to regenerate the polymer using ethyl acetate. The HPLC-MS quantification of the ethanol extract and eluted samples was performed. It showed that 1 mL of the dried and reconstituted in hexane ethanol extract contained 5.9 mg of artemisinin. The fraction eluted with hexane: 50% IPE eluent contained 55% of the loaded amount of artemisinin (3.3 mg) and fraction eluted with methanol contained 6.5 mg (110% of loaded artemisinin).

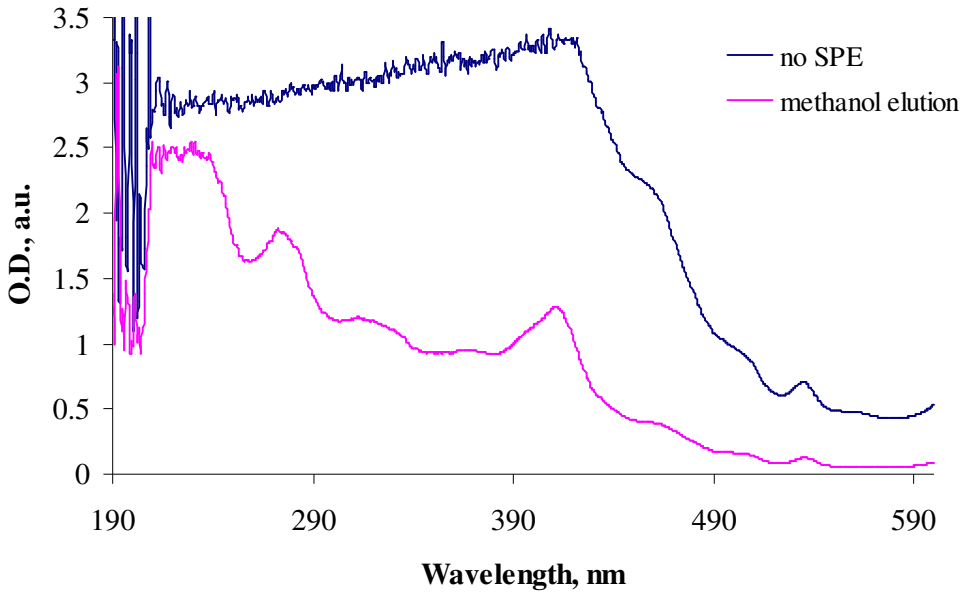

Figure 17. The chromatograms of the non-purified ethanol extract and fraction eluted using 1 mL of methanol.

Further optimisation of SPE protocol for artemisinin purification from ethanol extract

In order to optimise the SPE protocol for purification of artemisinin from ethanol extract following controls were made:

- the artemisinin concentration in the ethanol extract was quantified as 5.79 mg mL^{-1} using HPLC-MS;
- 10 mL of ethanol extract were dried and reconstituted (1:1, v/v) in 10 ml of hexane. Before loading hexane solution was further diluted 10 times in hexane. After the dilution the artemisinin concentration in the loaded solution was quantified as 0.57- 0.7 mg mL^{-1}. Although the artemisinin solubility in hexane is almost 10 times lower than in ethanol, it was found that together with other compounds of ethanol extract it was possible to transfer artemisinin almost completely into hexane.

Following SPE was conducted and artemisinin in all fractions was quantified:

1. Loading: 10 mL of hexane containing 1 mL of ethanol extract reconstituted in hexane;

2. Washing: 2 mL of hexane;
3. Elution: 2x 1 mL of methanol;
4. Regeneration: 1 mL of ethyl acetate.

Table 9. Quantification of artemisinin during all SPE steps (ethanol extract).

SPE steps	Artemisinin, mg	Recovery, %
Ethanol extract (1 mL of ethanol extract, dried and transferred into hexane;1:10 in hexane), 10 mL	5.7	n/a
Filtrate, 10 mL	0.4	7
Washing, 2 mL	0.177	3.1
Elution 1 1st mL of methanol	5.13	90
Elution 2 2nd mL of methanol	0.05	0.8
Regeneration, 1 mL ethyl acetate	0.04	0.7

It was found that it is possible to recover artemisinin completely from the ethanol extract (Table 9) when transferred into hexane. Unfortunately, there were still impurities which were eluted during the methanol elution together with artemisinin. In order to increase the purity of the eluted sample different eluents were tested and artemisinin recovery was quantified. Among the tested eluents were hexane:15% THF, hexane:50% IPE and cyclohexane:50% IPE. It was found that hexane:50% IPE and cyclohexane:50% IPE produced more clear samples than methanol but the artemisinin recovery was not quantitative. The elution with hexane:15% THF eluent produced the sample with less impurities than methanol eluent and quite high recovery of the artemisinin (73%) (Table 10).

Table 10. The comparison of artemisinin recovery using different solvents as eluents. The amount of loaded artemisinin was quantified as 7 mg.

Eluents	**Hexane:15%THF**		**Hexane:50% IPE**		**Cyclohexane:50% IPE**	
	Art, mg	**Rec., %**	**Art, mg**	**Rec., %**	**Art, mg**	**Rec, %**
E1 1st mL	2.25	31.4	0.97	13.5	1.03	14.4
E2 2nd mL	1.9	26.5	0.77	10.7	1.23	17.2
E3 3d ml	1.09	15.2	0.8	11.2	1	13.9
Total	5.24	73	2.54	35.4	3.26	45.5

Conclusions

1. Based on the results of computational modelling 4 functional monomers were selected for the polymer preparation. It included DEAEM, MBAA, AMPSA and TFMAA monomers.
2. Polymers based on these monomers were prepared and tested.
3. The HPLC-MS method for quantification of artemisinin was developed. It was found that the mass-spectrometry could provide a very reliable quantification method for monitoring of artemisinin in the SPE fractions.
4. MBAA-based polymer demonstrated the highest binding and was selected for the future study.
5. Capacity of the MBAA-based polymers towards the artemisinin in model solution (hexane) was evaluated as 120 mg per g of resin.
6. Capacity of the MBAA-based polymers towards artemisinin in plant extract (hexane) was estimated as 65- 70 mg of artemisinin per g of the resin.
7. The hexane could be used for the washing step. Based on HPLC-MS detection it did not remove artemisinin from the polymer but removed some impurities.
8. Since the elution should be in balance between the minimal removal of impurities and maximal recovery of artemisinin it was found that hexane:15 % THF is excellent recovery solvent, providing 4 times purification for artemisinin. Another very effective solvent was methanol; it was possible to elute the artemisinin with x10 times pre-concentration which resulted in the crystallisation of the compound. Unfortunately, the sample purity was lower than with hexane:15% THF because some yellow compounds were removed during the elution as well. Nevertheless the methanol elution is very interesting and should be considered in the future in combination with some other sample purification steps.
9. It was noticed that big quantity of the impurities was staying on the polymer and was removed only during the regeneration step (concentrated ethyl acetate or THF);
10. Polymer is stable. Although it was shown that it is possible to regenerate and re-load at least 10 times, it is very likely that it would be possible to regenerate for much longer;

11. The optimisation of purification of artemisinin from the ethanol extract was made. The ethanol extract was dried and reconstituted in hexane. It was found that the polymer could retain similar quantity of the artemisinin from reconstituted in hexane ethanol extract as from original hexane extract (60-70 mg per g of the polymer). The SPE of the reconstituted in hexane ethanol extract using a MBAA-based polymer demonstrated 73% of the artemisinin recovery together with substantial reduction in the level of impurities.

Future work

The future work in our opinion should include further optimisation of purification process for commercial samples using industrial volumes and larger size of columns. It would be interesting to combine our materials with other techniques such as ionic liquids used by other partners. There would be need as well to optimise polymer purification stage with following crystallisation.