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The impact of impurities in various crude *A. annua* extracts on the analysis of artemisinin by liquid chromatographic methods

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Abstract

Analysis of *Artemisia annua* extracts by liquid chromatographic methods has traditionally been complicated by the presence of significant quantities of impurities. It has been observed that these impurities often remain as a solid residue after sample reconstitution, but the possibility of artemisinin remaining entrained within this waxy layer has not been detailed in the literature. This investigation found that *A. annua* extract impurities have a critical impact on the quantification of artemisinin by liquid chromatographic methods. Extended sample reconstitution times of up to 24 hours are required in order for the mobile phase (acetonitrile) to penetrate the residue and solubilise the artemisinin contained within.

Extracts produced using ethyl acetate, hexane-ethyl acetate (95:5, v/v), hexane and ethanol were examined in the study. Extended residue reconstitution times resulted in a significant increase in the number and concentration of impurities in the mobile phase, requiring the development of a new HPLC-UV analytical method to exact adequate separation of artemisinin for quantification. The solvent selectivity and capacity for each of the solvent extraction approaches was then determined using the new reconstitution and HPLC-UV methods.

Keywords *Artemisia annua,* Artemisinin, Extract Reconstitution, HPLC-UV, Malaria, Selectivity

1. Introduction

Malaria remains one of the world's most prevalent diseases, with an estimated 500 million cases worldwide each year that result in over one million deaths [1]. Eighty percent of all deaths attributed to malaria occur in Sub-Saharan Africa, particularly in children under the age of five [2]. Due to the scale of the disease, international efforts are underway to significantly decrease the death rate by 2015, with an overall vision of the complete eradication of the disease thereafter [3]. Artemisinin-based combination therapies (ACTs) are currently the most effective treatment available for *Plasmodium falciparum* malaria, despite recent reports of region-specific parasitic resistance [4].

Artemisinin, a sesquiterpene lactone with an endoperoxide bridge, is the critical starting material for conversion to Active Pharmaceutical Ingredient (API) in all World Health Organisation (WHO) approved ACTs, and is primarily obtained through a solid-liquid extraction of *A. annua* L. leaves. Traditional solvents used for extraction include hexane, ethanol, petroleum ether, chloroform and toluene. More advanced extractions involve the use of supercritical CO_2 [5-7], ionic liquids [1], HFC-134a [1] and microwave assisted extraction (MAE) [8], which have all reported high efficiencies when compared to conventional extraction techniques. However, many of these technologies remain beyond the technical and capital reach of most producers in developing countries. Potential difficulties in the scale-up of newer technologies may also result in their performance falling short of conventional procedures (once optimised).

For smaller producers, the choice between traditional extraction solvents is complicated by the large number of conflicting reports in the literature [1]. Many experiments that compare extraction solvents use techniques such as soxhlet extraction that are not representative of those utilised in industry and which may lead to misleading conclusions. In addition, the solvent to leaf proportions used are often so high as to make subsequent artemisinin recovery through crystallisation a prohibitively energy-intensive process on an industrial scale. However, the elucidation of actual industrial procedures is extremely difficult as they are considered commercially sensitive, which severely hinders the appraisal of different extraction parameters on process efficiency. This is compounded by the array of different methods for recovering and purifying the artemisinin from extracts, whose efficiency is intrinsically linked to the initial extraction procedure.

Production process optimisation requires simple, robust and ideally rapid analytical techniques that can inform on process efficiency under varying parameters within a producer's frame of work. For this purpose, analysis of artemisinin has been undertaken using high performance liquid chromatography (HPLC) with ultraviolet detection (-UV) [9-13], evaporative light scattering detection (-ELSD) [12, 14] and refractive index detection (-RI) [11]. In addition, thin layer chromatography (TLC) [15], nuclear magnetic resonance (NMR) [10, 16], liquid chromatography - mass spectrometry (LC-MS) [10, 17], and gas chromatography with both mass spectrometry (GC-MS) [18] and flame ionisation detection (GC-FID) [14] methods have been developed, among others. Of all techniques, HPLC-UV likely offers the most favourable balance between reliability, simplicity of operation and capital cost, but remains the most contentious approach in several aspects.

The important evaluation of different analytical methods for the quantification of artemisinin in crude leaf extracts made by Lapkin *et al.* [11], and funded by Medicines for Malaria Venture (MMV), was necessary because the HPLC-UV method given in the WHO Monograph and the International Pharmacopoeia cited by Lapkin *et al.* [11] is intended for the analysis of API or starting materials. Many researchers maintain that the lack of a chromophore for artemisinin makes UV detection unsuitable [9, 12, 16, 19]. In these cases, derivatisation to a UV-absorbing compound (Q260) is undertaken by hydrolysis with sodium hydroxide, followed by acidification which increases the complexity of the procedure. On the other hand, those that choose to analyse artemisinin without prior derivitisation are met with conflicting reports of the optimal wavelength.

The WHO Monograph states that detection should be undertaken at around 216nm, but it has been suggested that this wavelength introduces a high bias to the estimation of impurity concentrations [10]. The study by Lapkin *et al.* [11] examined a UV range of between 205 and 215nm and determined the maximum absorbance in this range to occur at 210nm. Stringham *et al*. [10] also noted that improved sensitivity was achieved when analysing at 210nm instead of 216nm. However, it was previously determined that the maximum UV absorbance occurs at 192nm and that a sharp decay in response should be expected as the wavelength was increased [12].

It has been suggested that HPLC-UV analysis is inappropriate for crude artemisinin extracts as the peak response can be difficult to detect among highly-absorbing impurities [11]. This

conclusion was supported by a chromatogram of an ethyl acetate extract but the chromatograms for the hexane, acetonitrile or chloroform extracts were not presented. As no reference to pure ethyl acetate extractions can be identified in the literature and acetonitrile would be unacceptably hazardous to use on a large scale [1], these findings do not necessarily invalidate HPLC-UV analysis of industrial extracts. The modified WHO Monograph method developed by Lapkin *et al.* [11], hereon referred to as "Modified HPLC Protocol", was not applied to ethanol extracts and although a method does exist for their analysis it involves the prior derivatisation of artemisinin [9].

One final difficulty in applying any analytical technique that relies on liquid chromatography is the method of sample reconstitution in the mobile phase. Whilst it is acknowledged that several impurities in artemisinin extracts are poorly soluble in acetonitrile, the impact of their presence on the reconstitution of artemisinin is not known. If these impurities hinder the solubilisation of artemisinin, the detectable artemisinin content will be highly dependent on the particular method of reconstitution. Thus the artemisinin content analysed may not be a true representation of the artemisinin content extracted. Precise details for reconstitution are not presented in analytical investigations and may therefore cause difficulties in method repeatability.

There were a number of aims to this investigation, with the primary aim to determine the impact that impurities have on the reconstitution of artemisinin from extract residue into acetonitrile as this has implications in all liquid chromatographic methods of extract analysis. This was undertaken by altering the contact duration of the mobile phase with extract residue prior to HPLC-UV analysis. An attempt was made to simulate the recent HPLC investigation of ethyl acetate extraction [11]. However, some parameters had to be estimated as the extraction temperature and duration were not presented, and it is not known if mixing was employed. Extractions with hexane, ethanol and a hexane-ethyl acetate (95:5, v/v) mixture were also undertaken as these solvents are commonly used in industry. It is claimed in the literature that the hexane-ethyl acetate (95:5, v/v) mixture is capable of extracting significantly more artemisinin than a pure hexane extraction [20]. For these extractions, the hexane fraction from petroleum and 96% aq. ethanol were used as it was felt that these solvents would be more representative of those used for extraction by industrial producers. With the difficulties identified by [11] in applying the Modified HPLC Protocol to crude artemisinin extracts, an alternative in-house method of HPLC-UV analysis is presented, and

the method was applied to assess solvent selectivity and the extent of artemisinin extraction under the conditions investigated.

2. Experimental

2.1. Materials and chemicals

Dried *A. annua* L. leaf was supplied by Afro Alpine Pharma Ltd. (Kabale, Uganda) and had already been milled to 4mm. Artemisinin standard (>98%) was obtained from Sigma Aldrich (Poole, UK). Ethyl acetate, acetonitrile and methanol were of HPLC gradient grade and were obtained from Fisher Scientific (Loughborough, UK). The hexane fraction of petroleum (95%; boiling range 65.5-70.0°C) and 96% aq. ethanol were also purchased from Fisher Scientific.

2.2. Extract Production

Extractions were undertaken using ethyl acetate, hexane, ethanol and a hexane-ethyl acetate (95:5, v/v) mixture. Each method of extraction was performed in triplicate and involved contacting 10g of leaf with 100±1mL of solvent for a period of four hours at room temperature (17±1°C). To avoid any additional maceration of the leaves, the mixture was agitated on a mixer table operating at approximately 150rpm. An additional, static extraction with ethyl acetate was undertaken to assess the impact of mixing.

After extraction, the miscella was strained through two layers of muslin fabric to separate the majority of the leaf matter before being vacuum filtered through 0.45μm. It was noted that approximately 25% of the miscella remained entrained in the leaf for each of the solvents used; a phenomenon common to industry. Each extract was then divided into eight 5mL aliquots, which were left uncovered in 7mL vials at room temperature for evaporation to dryness to occur. The residue weight was recorded. These vials were used to determine the extent of artemisinin extraction, the influence of reconstitution method on sample analysis, the solvent selectivity and inter-batch variability.

2.3. Residue reconstitution

All residues were reconstituted with 2.5mL of mobile phase. Of the eight vials from each extract, four were reconstituted in an acetonitrile-water-methanol (50:30:20, v/v/v) mixture, and the remaining four were reconstituted in pure acetonitrile. Each of the four vials was subjected to a different method of reconstitution; 30 seconds on a vortex (2800rpm) or mixing at approximately 350rpm for durations of 10 minutes, 30 minutes and 24 hours. After reconstitution, the solution was filtered through a 0.2μm PTFE membrane prior to HPLC-UV analysis.

2.4. HPLC-UV analysis

Analysis was undertaken using an Agilent 1260 HPLC system (Berkshire, UK) equipped with an Agilent multiple wavelength detector (MWD). Separation was achieved using a 250mm×4.6mm×5μm SunFire C18 column (Waters, Hertfordshire) at a solvent flow rate of 1mL min^{-1}. Two methods were utilised; the Modified HPLC Protocol method [11], and an in-house method developed for crude extracts. Although the Modified HPLC Protocol calls for a Betasil C18 column of the same dimensions, a comparable retention time for artemisinin (7.3 minutes) was achieved using the SunFire column. The in-house method utilises an isocratic mobile phase of acetonitrile-water (50:50, v/v) at 25°C for 25 minutes, followed by an acetonitrile-water (80:20, v/v) wash for six minutes and a subsequent re-equilibration period of six minutes. UV detection was undertaken simultaneously at 192, 200, 205, 210 and 215nm, with 210nm used for quantification in line with the Modified HPLC Protocol. Both methods used a 20μL injection loop.

A six-point calibration curve was produced by mixing or diluting two artemisinin stock solutions of 2mg mL^{-1} and 1mg mL^{-1} in the appropriate mobile phase. Good linearity was observed in the range 50-2000μg mL^{-1} ($R^2 > 0.999$). When not in use, stock solutions were stored at 4±0.5˚C.

3. Results and discussion

3.1. HPLC-UV analysis of artemisinin standard

Before proceeding with the analysis of crude *A. annua* extracts, the absorbance of artemisinin at several wavelengths was investigated. Artemisinin standard (1000µg mL^{-1}) was analysed at 192, 200, 205, 210 and 215nm over a period of seven days (n=5) using both the in-house and Modified HPLC Protocol mobile phases. The area response of artemisinin at each wavelength can be observed in Fig. 1. Detection at wavelengths below 192nm was not possible with the UV detection system used.

No significant degradation of artemisinin was observed in either pure acetonitrile or acetonitrile-water-methanol (50:30:20, v/v/v) over the seven day period. This was verified by overlaying five calibration curves generated over a period of seven days (Fig. 2), which still demonstrated excellent linearity ($R^2 = 0.9993$) in the range 50-2000µg mL^{-1} in acetonitrile. A similar result was obtained for samples stored in an acetonitrile-water-methanol (50:30:20, v/v/v) mixture.

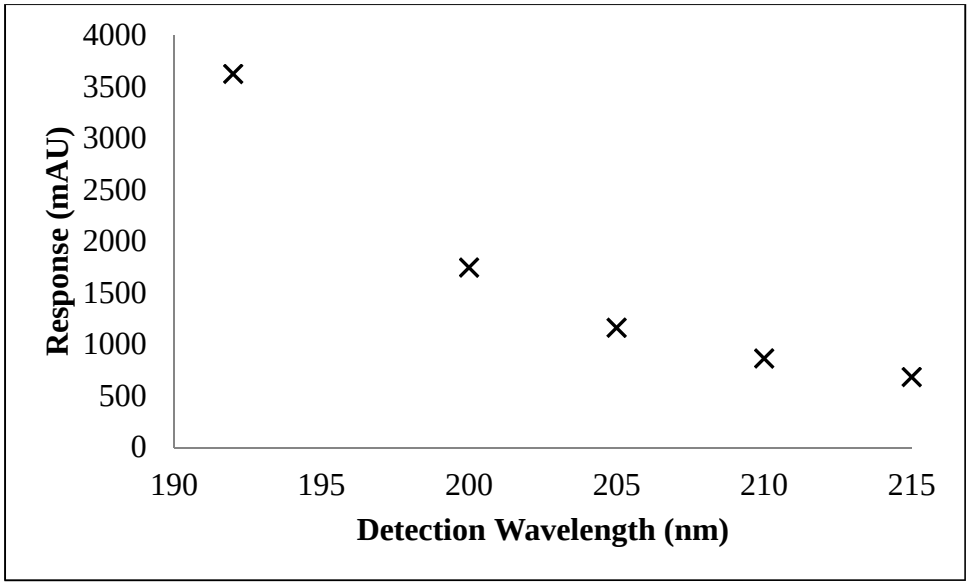

Fig. 1: Average (n=5) signal intensity of artemisinin standard (1000µg mL^{-1} in acetonitrile) as a function of detection wavelength.

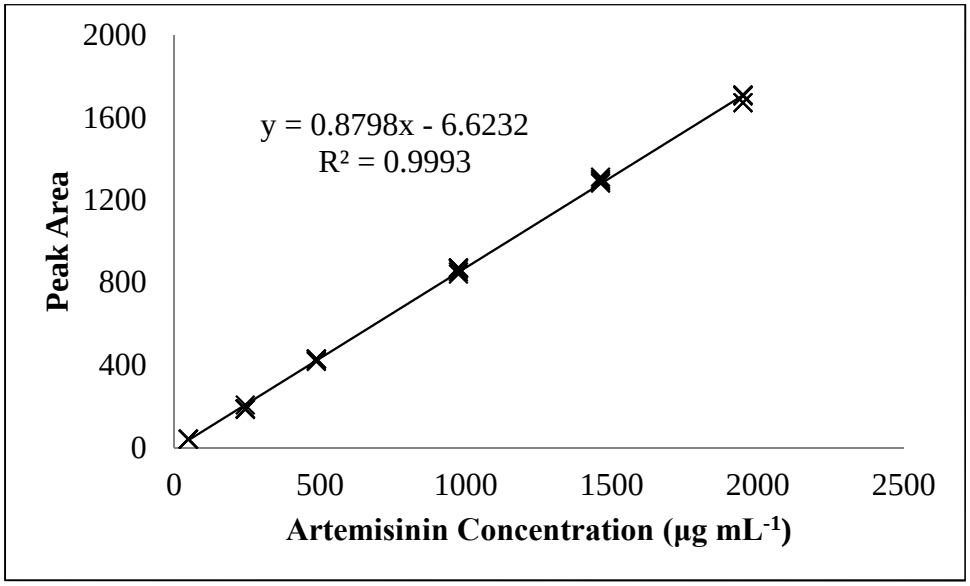

Fig. 2: Combined HPLC-UV calibration curve of analytical runs undertaken over a period of seven days (n=5). Samples stored at 4±0.5°C in pure acetonitrile.

From Fig. 1, it can be observed that the UV absorbance of artemisinin is at a maximum at 192nm and decreases with increasing wavelength within the tested range. Whilst these results support the findings of Ferreira and Gonzales [12], they do not correlate well with the Modified HPLC Protocol [11] as the results in Fig. 1 would suggest a 34.7±0.1% stronger response at 205nm than at 210nm. However, analysis at 192nm did introduce a higher degree on baseline noise (Fig. 3) and the limit of detection at 192nm, set at three times the baseline noise height, was found to be 15µg mL^{-1}, whilst that at 210nm was 4µg mL^{-1}. As the Modified HPLC Protocol verified analysis at 210nm, and detection at 192nm introduces increased baseline noise, all subsequent quantification was undertaken with detection at 210nm. However in practice, the artemisinin content present in sample extracts is such that a limit of detection at 15µg mL^{-1} would not negate quantification. Indeed all sample extracts herein were detected at multiple wavelengths between 192nm and 210nm and demonstrate that use of 192nm is acceptable.

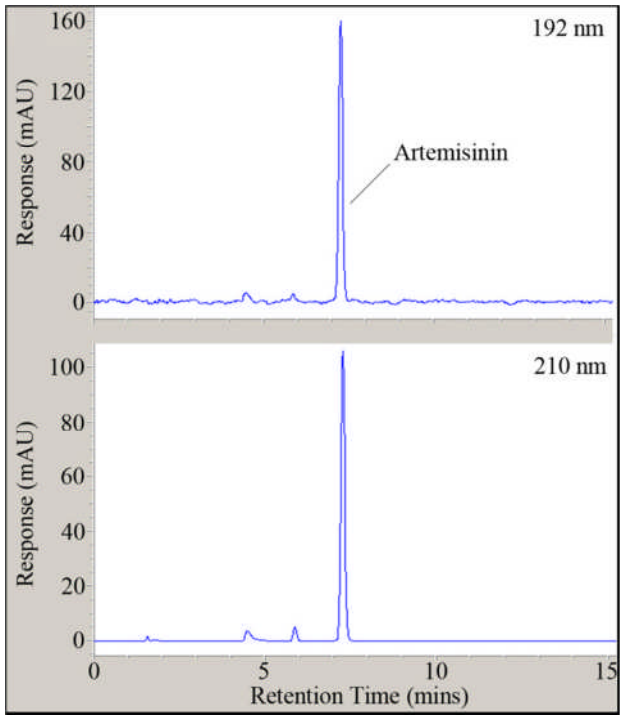

Fig. 3: Comparison of artemisinin standard (1000µg mL^{-1}) HPLC-UV chromatograms at 192 and 210nm when analysed using the Modified HPLC Protocol method; Mobile phase acetonitrile-water-methanol (50:30:20, v/v/v), SunFire C18 250mm×4.6mm×5µm at column flow rate 1mL min^{-1} and column temperature 40°C.

3.2. HPLC-UV analysis of crude extracts

The two HPLC-UV methods were applied to the crude *A. annua* extracts. Chromatographs for the agitated ethyl acetate, hexane and ethanol extractions are presented in Fig. 4 for the Modified HPLC Protocol method and Fig. 5 for the in-house method. In both cases, the chromatographs are for residues reconstituted by mixing in the mobile phase for a period of 30 minutes prior to HPLC-UV analysis. Significant co-elution of artemisinin with impurities is observed in Fig. 4 for both the ethyl acetate and hexane extractions using the Modified HPLC Protocol method, making quantitative analysis impossible.

This co-elution, however, was not observed for samples reconstituted for a period of 30 seconds. Due to the low contact time, many of the extract impurities were not in sufficient concentration to be detected by HPLC-UV. The chromatographs for a mixed ethyl acetate extract reconstituted both for a period of 30 seconds and for 30 minutes prior to HPLC-UV

analysis is presented in Fig. 6. It was additionally found that the Modified HPLC Protocol was not suitable for samples reconstituted in acetonitrile, though there was some change to the impurity profile around artemisinin. A chromatograph illustrating this effect is included in Fig. 6.

The acetonitrile-water (50:50, v/v) method demonstrated separation of artemisinin from impurities for all extracts examined (Fig. 5). It can also be observed that the response of artemisinin in Fig. 5 is sufficiently strong in relation to the impurities that it cannot be overlooked and can be used to reasonably quantify artemisinin.

Due to the difficulties in applying the Modified HPLC Protocol method due to co-elution, it was determined that subsequent analysis should be undertaken using the in-house HPLC-UV method.

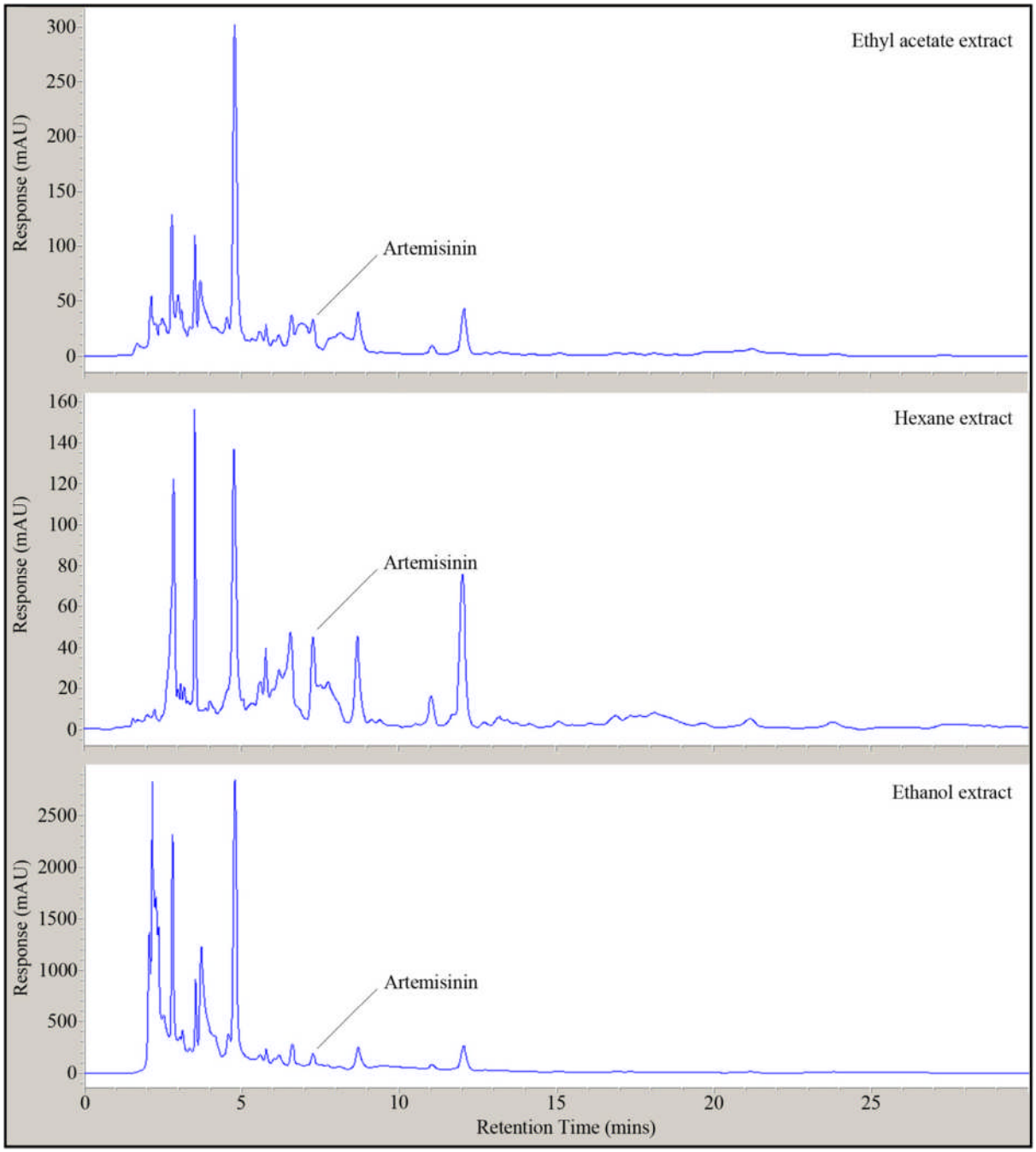

Fig. 4: HPLC-UV analysis of crude *A. annua* extracts produced using ethyl acetate, hexane and ethanol. Modified HPLC Protocol method; mobile phase of acetonitrile-water-methanol (50:30:20, v/v/v), SunFire C18 250mm×4.6mm×5µm at column flow rate 1mL min^{-1} and temperature 40°C, 210nm detection. Due to co-elution of artemisinin with impurities, the concentration of artemisinin in the extracts could not be determined.

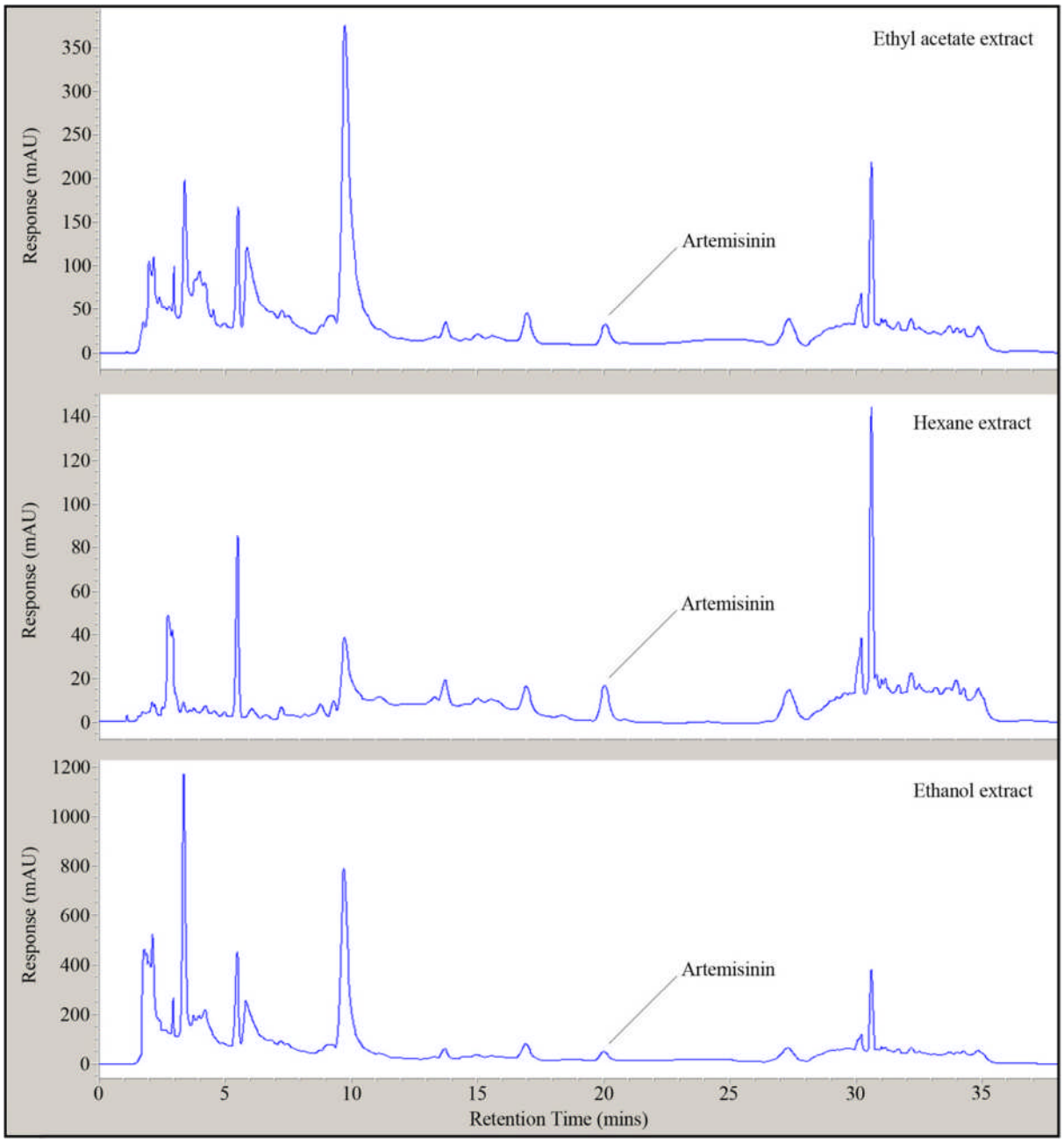

Fig. 5: HPLC-UV analysis of crude *A. annua* extracts produced using ethyl acetate, hexane and ethanol. In-house method; mobile phase of acetonitrile-water (50:50, v/v) with an acetonitrile-water (80:20, v/v) wash after 25 minutes. SunFire C18 250mm×4.6mm×5µm at column flow rate 1mL min^{-1} and column temperature 25°C, 210nm detection. The detected concentration of artemisinin is 608, 432 and 914µg mL^{-1} for the ethyl acetate, hexane and ethanol extracts respectively.

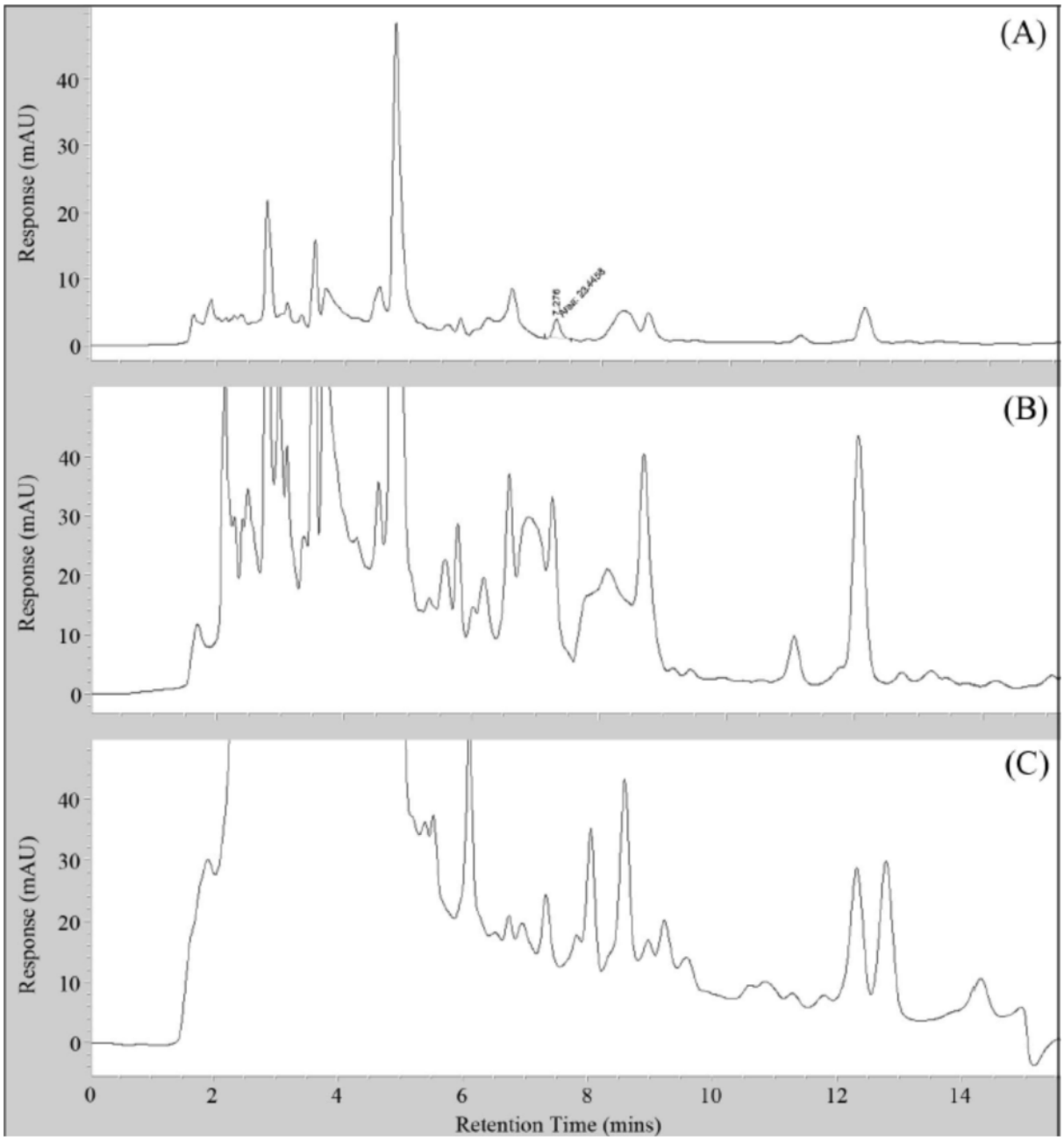

Fig. 6: Modified HPLC Protocol analysis of crude *A. annua* extracts produced using ethyl acetate with mixing. (A) Residue reconstituted in acetonitrile-water-methanol (50:30:20, v/v/v) mixture for 30 seconds on a vortex. (B) Residue reconstituted in acetonitrile-water-methanol (50:30:20, v/v/v) mixture with 30 minutes of mixing. (C) Residue reconstituted in acetonitrile with 30 minutes of mixing.

3.3. Effect of reconstitution method

Crude *A. annua* extracts contain significant quantities of impurities that are observed to remain as a wax-like residue when reconstituting samples in acetonitrile for liquid

chromatographic analysis. The potential for these residues to hinder the reconstitution of artemisinin, and therefore prevent its subsequent detection and accurate quantification, has not been examined in the literature. As artemisinin was observed to co-elute with impurities when analysing extracts by the Modified HPLC Protocol method (Fig. 4), quantitative analysis of the artemisinin content of extract residues could only be undertaken using the in-house analytical method (Fig. 5). The quantity of artemisinin obtained from the leaf batch extractions is presented in Fig. 7 as a function of extraction solvent and sample reconstitution method prior to HPLC-UV analysis.

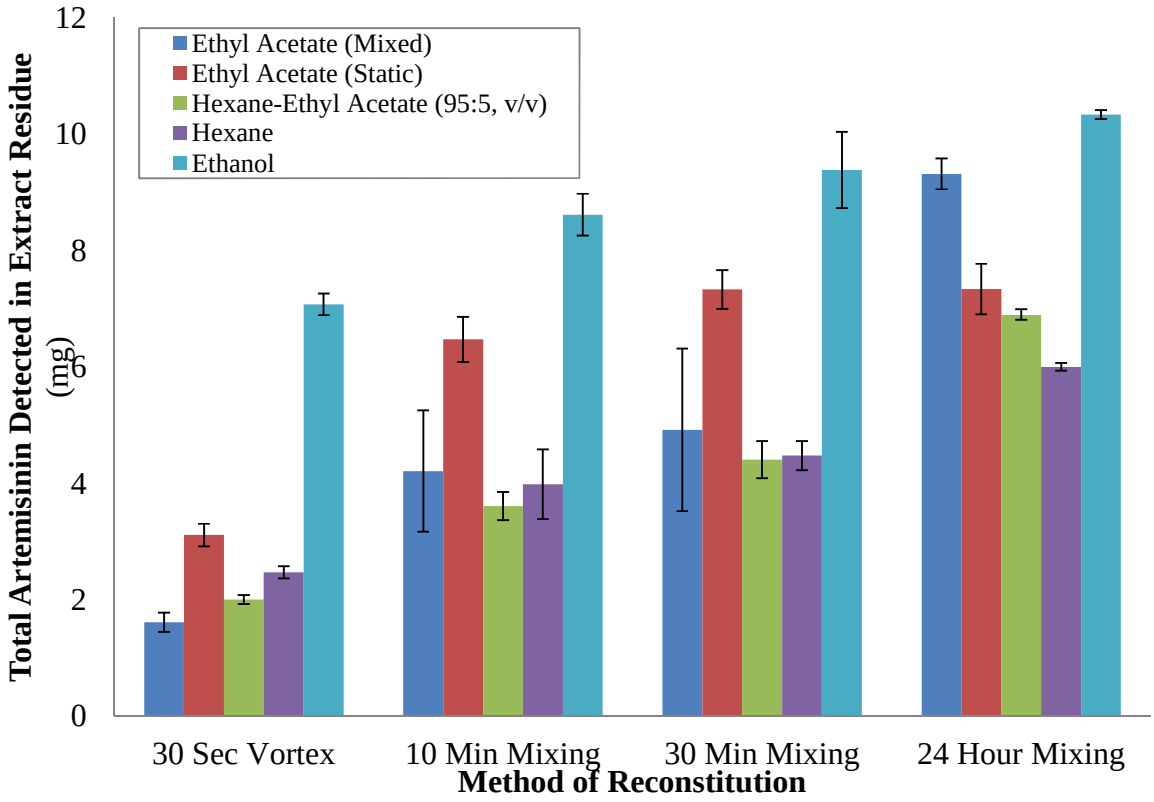

Fig. 7: The total amount of artemisinin detected by HPLC-UV in *A. annua* extracts as a function of extraction solvent and method of reconstitution into acetonitrile. Extracts produced by contacting 10g leaf with 100mL solvent mixture for four hours at room temperature (17±1°C) with mixing (unless otherwise stated). 5mL of extract evaporated to dryness and reconstituted with 2.5mL of acetonitrile using the four methods listed (n=3).

A relationship is demonstrated in Fig. 7 between the duration of sample reconstitution and the subsequent, analytically-determined concentration of artemisinin in the extract residue. In all cases, an increased duration of reconstitution resulted in the detection of more artemisinin.

This is likely due to the presence of significant quantities of impurities such as waxes that are insoluble in acetonitrile and act as a barrier to prevent acetonitrile contacting and solubilising artemisinin contained within the residue. With increased contact duration with the mobile phase, the solid wax-like impurities were observed to fragment and become bleached of colour. After 24 hours, the residue was detached from the sides of the vial, allowing acetonitrile to solubilise the previously entrained artemisinin.

This hypothesis is further supported by the observation that inter-batch variability is lowest for samples reconstituted by 30 seconds on a vortex and those reconstituted for a period of 24 hours. In the former case, the short contact duration will ensure that only artemisinin on the surface of the residue is dissolved in the mobile phase. As the residue creates a relatively smooth coating on the glass vials, the variability in the quantity of artemisinin solubilised between samples will be minimised. For the intermediate contact durations, the solubilisation of artemisinin will be determined by the penetration of solvent into the residue, which will be much more variable. After 24 hours the penetration of solvent into the residue is most complete and the detected artemisinin concentration is more representative of that in the actual extract.

One can also compare the analytically determined concentration of artemisinin in the ethyl acetate extracts, both with and without mixing. It is logical that the application of mixing would assist in artemisinin extraction but the data from extracts reconstituted for 30 minutes or less actually suggest that static extraction is superior. It is only when the samples have been reconstituted for 24 hours that the benefit of mixing becomes evident and there are two likely mechanisms responsible for this. Firstly, the total extract residue quantity (artemisinin and impurities) will increase when mixing is employed, thus increasing the total depth of the residue layer that must be penetrated by the mobile phase. Secondly, it is possible that the proportion of artemisinin to impurities is decreased when mixing is used. Assuming comparable residue densities, this would mean that for an equal (incomplete) penetration depth of acetonitrile into the residue from both mixed and static extractions, more artemisinin will be reconstituted from the static extract residue.

The finding is significant as it has implications in all analytical techniques utilising liquid chromatography, regardless of the detection method employed, as reconstitution methods will have a direct and appreciable effect on the detected concentration of artemisinin. The effect

will be greatly amplified when comparing results from two or more different investigations, where the approach to sample reconstitution is likely to vary more significantly than within a single study. In order to obtain accurate results of the true concentration of artemisinin in extracts, reconstitution must be undertaken over an extended period of time.

Furthermore, the findings could have more far-reaching impacts in both analysis (such as TLC) and purification involving adsorption mechanisms. For example, when using a silica gel adsorbent for extract purification, waxy deposits are known to build up on the substrate. Translating from the findings herein, artemisinin will be entrained within the deposits and a relatively rapid elution method to recover artemisinin from the column may not be able to fully penetrate this layer of waxes. The result is sub-optimal recovery of artemisinin from the purification stage, with artemisinin eluting as a broad smear that leads to a dilute eluate. It may be possible to address this issue by increasing the elution times, and thus increase the contact duration between the solvent and the waxy deposits, allowing for greater solvent penetration.

Reconstitution of samples for a period of 24 hours for analysis, however, is impractical, though no method of enhanced reconstitution has been investigated in this study A potential way to reduce the impact of the reconstitution times is to correlate the results obtained from the most convenient reconstitution method to those that would be obtained from the most accurate method. This can be achieved by dividing the quantity of artemisinin reconstituted in a period of 24 hours by that which is reconstituted in 30 seconds, to obtain a scaling factor. Table 1 lists the calculated scaling factors for each of the extractions undertaken and it can be observed that they can be applied with relatively high precision (CV < 3.3%) for all but the ethyl acetate extracts. Note that the coefficient of variation presented is for inter-batch variability as each sample in the triplicate analysis was obtained from separate batches. Intra-batch accuracy of results would inevitably be superior due to the reduced number of variables involved.

Table 1: Scaling factors obtained when correlating the amount of artemisinin reconstituted from *A. annua* extract residues into acetonitrile over a 30 second period of vortexing (2800rpm) to that obtained from 24 hours of reconstitution using mixing (350rpm).

Leaf Extraction Solvent	Scaling Factor[a]	CV[b] (%)
Ethyl Acetate (Mixed)	5.77	8.69
Ethyl Acetate (Static)	2.35	9.40
Hexane-Ethyl Acetate (95:5, v/v)	3.44	3.26
Hexane	2.43	3.14
Ethanol	1.46	3.08

[a] (Artemisinin reconstituted after 24 hours / Artemisinin reconstituted after 30 seconds)

[b] CV ≡ Coefficient of variation; the standard deviation in scaling factor expressed as a percentage of the mean (n=3) scaling factor.

3.4. Artemisinin extraction and solvent selectivity

The total amount of artemisinin obtained from each extraction approach can be used to determine the solvent selectivity; the weight proportion of artemisinin to impurities in the extract. The results presented in Table 2 are based on residue reconstituted for a period of 24 hours with mixing in acetonitrile prior to HPLC-UV analysis.

Table 2: The total amount of artemisinin extracted from *A. annua* using various solvents for a period of 4 hours at room temperature (n=3), and the purity of the artemisinin within the extract.

Leaf Extraction Solvent	Extract Artemisinin Purity		Total Artemisinin Extracted	
	Mean (wt%)	CV (%)	Mean (mg)	CV (%)
Ethanol	16.91	0.74	10.34	0.08
Ethyl acetate (no mixing)	12.66	1.86	7.34	0.43
95:5 hexane:ethyl acetate	11.29	1.32	6.90	0.09
Ethyl acetate (mixing)	10.71	4.24	9.32	0.27
Hexane	9.82	1.10	6.00	0.07

Table 2 demonstrates that the optimal extraction under these conditions was achieved with ethanol, both in terms of the total amount of artemisinin extracted and the solvent selectivity. Hexane extracted the least amount of artemisinin and also demonstrated the lowest selectivity. These results contrast with the trends quoted in the literature [21], where hexane was shown to exhibit the highest selectivity and ethanol the lowest. Whilst ethanol should be expected to achieve a greater artemisinin extraction due to the increased solubilisation capacity over hexane, it was claimed to also extract a significantly higher proportion of impurities.

In line with the literature claims, the addition of 5% v/v ethyl acetate to hexane was able to increase the extent of artemisinin extraction and overall selectivity over a pure hexane extraction. The increase, however, falls far short of the two-fold increase reported in [20]. It can also be observed that agitation increased the total amount of artemisinin extracted by ethyl acetate over the four hour period, but resulted in a reduced selectivity due to the extraction of a greater proportion of impurities.

However, no attempt was made to recover artemisinin from the sample extracts and the influence of extraction conditions (temperature, solvent to leaf proportions and extraction duration) were not examined. These parameters would need to be addressed before an optimal extraction approach can be decided, but it has been demonstrated that the HPLC-UV method developed herein may be applied to assess the quality of extracts.

4. Conclusions

The UV response of underivatised artemisinin was found to be greatest at 192nm and decayed significantly with increasing wavelength over the range of wavelengths investigated. This finding supports research by Ferreira and Gonzales [12] but is in contradiction to the Modified HPLC protocol [11] which observed a greater response at 210nm than at 205nm. It was identified, however, that analysis at 192nm was accompanied by an increase in the baseline noise which subsequently caused an increase in the limit of detection from 4μg mL^{-1} to 15μg mL^{-1}.

The recent Modified HPLC Protocol for the analysis of artemisinin with UV detection was not recommended for crude *A. annua* extracts [11]. This was due to the scale of the impurity

UV response, which was liable to obscure the artemisinin peak. In this investigation, the Modified HPLC Protocol method was applied to a variety of crude extracts and, although the UV response of artemisinin was clearly visible, the co-elution of impurities made quantitative analysis impossible for ethyl acetate and hexane extracts. Adequate separation of artemisinin from impurities could be achieved with ethanol extracts.

An in-house HPLC-UV analytical method that is applicable to ethyl acetate, hexane, ethanol and hexane-ethyl acetate (95:5, v/v) extracts has been demonstrated. Whilst artemisinin elutes approximately 12 minutes later than the Modified HPLC Protocol method, thus extending the analytical runtime, a significantly improved separation of artemisinin from impurities is achieved. The proposed method does not require derivatisation of artemisinin and is therefore a simplified approach over many other analytical procedures reported in the literature.

This study demonstrates that the method of extract reconstitution prior to HPLC-UV analysis has a critical impact on the detected concentration of artemisinin. Many co-extracts exhibited poor solubility in acetonitrile and acted as a barrier, preventing the solubilisation of artemisinin contained within the residue. Prolonged reconstitution in pure acetonitrile is required to fully solubilise the artemisinin into the mobile phase for analysis. Correlations relating the quantity of artemisinin dissolved after 30 seconds of rapid mixing to that dissolved over 24 hours can be made with a precision greater than ±3.3% for ethanol, hexane and hexane-ethyl acetate (95:5, v/v) extracts under the extraction parameters investigated. Pure ethyl acetate extracts cannot be predicted with the same precision, though are unlikely to be encountered in industry because there are no reports of its use on an industrial scale in the literature.

Ethanol was found to exhibit both the highest selectivity and extraction capacity for artemisinin from the solvents and extraction conditions examined, whilst hexane exhibited the lowest selectivity and extraction capacity. This is in direct contradiction to the trend frequently discussed in the literature. The efficiency of extraction solvents needs to be investigated under a range of extraction conditions that are representative of industrial processes to allow for an informed decision regarding the optimum extraction procedure.

5. Acknowledgements

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