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Comparative Quantitative Analysis of Artemisinin by Chromatography and qNMR

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ABSTRACT:

Introduction – Since the discovery of artemisinin in the 1970s, many techniques based on diverse chromatography techniques have been developed to detect and quantify this important antiplasmodial compound. The accurate quantification of this compound in the *Artemisia annua* plant material is mainly needed for breeding purposes in order to cultivate higher yielding varieties. It is also important for the quality control of herbal preparations containing *A. annua* plant material.

Objective – To evaluate the most common validated quantification techniques (LC-MS, HPLC-ELSD and TLC) and compare the results to quantitative nuclear magnetic resonance spectroscopy (qNMR) in eight different *A. annua* samples collected from around the world.

Methodology – The leaf material were extracted according to standard procedures and analysed with the validated quantification techniques. For the qNMR analysis we did not employ a standard curve but instead used an internal standard (maleid acid) which is not chemically related to artemisinin.

Results – We found a significant difference between the results in this study. Compared with the qNMR results the HPLC-ELSD corresponded closely, followed by LC-MS. Quantitation with TLC led to an estimation range of –0.5 to +3.2 mg artemisinin/g of *A. annua*.

Conclusion – These results imply that qNMR, with the addition of an internal standard, can be used to quantify artemisinin in *A. annua* samples in a rapid and reproducible manner. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: *Artemisia annua*; artemisinin; chromatography; quantitative nucleur magnetic resonance spectroscopy

Introduction

Malaria is a vector-borne infectious disease caused by the protozoan *Plasmodium* parasites. It causes disease in approximately 515 million people and kills between 1 and 3 million people, the majority of whom are young children in Sub-Saharan Africa (Snow *et al.*, 2005). Currently the WHO recommends the use of artemisinin (ART) combination therapies to treat patients suffering from uncomplicated malaria. Artemisinin, which forms the backbone of this treatment regime, was isolated from a medicinal plant, *Artemisia annua* L. (Asteraceae), in the 1970s. Because of the chemical properties of this compound, mainly its low extinction coefficient, various detectors other than UV detection have been employed for quantitation, including high-pressure liquid chromatography–evaporative light scattering detection (HPLC–ELSD) (Avery *et al.*, 1999; Peng *et al.*, 2006; Liu *et al.*, 2007), thin-layer chromatography (TLC) (Koobkokkruad *et al.*, 2007; Widmer *et al.*, 2007; Marchand *et al.*, 2008) with chemical visualisation, liquid chromatography–mass spectroscopy (LC-MS) (Wang *et al.*, 2005; Van Nieuwerburgh *et al.*, 2006) and nuclear magnetic resonance (NMR) (Castilho *et al.*, 2008). Quantification with gas chromatography (GC) is also well known (Woerdenbag *et al.*, 1991; Sipahimalani *et al.*, 1991; Ferreira *et al.*, 1994; Peng *et al.*, 2006), but due to the thermal instability of ART, this technique measures the breakdown products of ART and is therefore an indirect quantification technique.

Most quantification techniques make use of the two-step sequence of (1) chromatographic separation of the complex mixture of components in the plant extract and (2) quantification of the separated components with the use of specific detectors. In qNMR analysis there is no need to separate ART from the other components in the extract (chemical shift peak overlap can be a problem in the accurate quantification of compounds—this is not the case for ART due to the unique chemical shift of the methine proton used for quantitation). Taking into account the variation occurring during the chromatographic separation step, this is one of the major advantages for the quantification of ART with qNMR analysis. Another advantage of qNMR analysis is that no standard curve is needed for the quantification of ART, and with the addition of an internal standard (not necessarily related to the target compound) and with the use of an appropriate relaxation delay, it is possible to accurately quantify target compounds (Kim *et al.*, 2003; Choi *et al.*, 2004; Pauli *et al.*, 2005). With the qNMR method the analysis time can also be considerably reduced. The main drawbacks of qNMR analysis are the lower sensitivity compared with other detection methods and the possibility that chemical shift peak overlap may occur with the target signal to be quantified. This will limit the use of qNMR as a quantification tool to samples with a relatively high ART

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concentration, as is found in the *A. annua* plant material. The unique low field chemical shift of the methine proton of ART makes qNMR the ideal tool for quantification of this compound in the plant material.

A validated qNMR method for the quantitation of ART using a standard curve was published recently. Our qNMR method makes use of an internal standard (not related to ART) without the need for a standard curve. The use of qNMR techniques and the application to natural products is excellently reviewed by Pauli *et al.* (2005). Our aim was to perform a comparative study of four recently published validated quantification methods for ART in the *A. annua* plant material (validation data not included).

All of the quantification methods were reproduced as closely as possible to quantify ART in eight samples collected from different parts of the world. An important factor to take into account is that the plant material used during our experiments is different from those used in the publications we are reproducing. This might lead to the possibility of peak overlap during the chromatographic separation step. We therefore had to adjust the mobile phases in certain cases in order to separate ART from other unknown compounds in the extracts. In addition we also had to adjust certain parameters on the detectors so as to achieve the highest possible sensitivity, as we do not have the exact same detectors (different models or from different suppliers) as reported in the publications. All of the adjusted parameters have been noted in the experimental section.

Experimental

Plant material

In total eight *A. annua* (Anamed A-3) samples were purchased from Anamed (Winnenden, Germany). These samples were collected by Anamed from different countries around the world. The samples were supplied with information pertaining to the country of origin, plant part, storage conditions and the year of harvest. Table 1 gives the information for each sample.

Chemicals

Artemisinin (>98% pure), anisaldehyde, trifluoroacetic acid, maleic acid and LC-MS grade mobile phase were purchased from Sigma-Aldrich (Steinheim, Germany). All organic solvents used for sample and standard solution preparation were of analytical grade, and the organic mobile phase for HPLC analysis were of HPLC grade and was purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Formic acid was purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands), and the deuterated solvent, CD_3OD, for NMR analysis was purchased from Andover (MA, USA). HPTLC silica gel 60 plates, cyclohexane and sulphuric acid were obtained from Merck KGaA (Darmstadt, Germany), Fluka (Buchs, Switzerland) and Boom Lab (Meppel, The Netherlands), respectively. Deionised water was obtained from the Department of Pharmacognosy at Leiden University.

Sample preparation

In total 24 samples were prepared for extraction (each of the eight plant samples in triplicate). Each sample was dried and ground by a mill grinder. The ground material (500 mg) was extracted by refluxing each sample with 50 mL of *n*-hexane at 75°C for 1 h (Peng *et al.*, 2006), after which the samples were filtered and dried using standard procedures. All the samples were re-dissolved in 10.0 mL of acetonitrile of which 1 mL was transferred to separate 1.5 mL vials for HPLC-ELSD, TLC and LC-MS analysis. The same extraction method was employed for the qNMR analysis. After filtration, however, the solvent volumes were reduced *in vacuo* after which the samples were transferred into vials and dried completely under nitrogen flow. An internal standard was prepared by dissolving 200 mg of maleic acid in 100 mL of methanol. Of this stock solution, 0.1 mL was added into each vial containing the *A. annua* extract. The samples were subsequently dried and re-dissolved in 1 mL of CD_3OD. Of this solution 0.8 mL was transferred into NMR tubes for further analysis. All samples were stored at −20°C until analysed.

HPLC-ELSD analysis

Three published methods were consulted and tested on our analytical system (Avery *et al.*, 1999; Peng *et al.*, 2006; Liu *et al.*, 2007). The method reported by Peng *et al.* (2006) was found to be the most suitable for our analytical equipment and was used to determine the concentration of ART in the samples. Six standard solutions were prepared by dissolving 2 mg of ART in 2 mL of acetonitrile (1 mg/mL). This standard solution was serially diluted to 0.5, 0.25, 0.125, 0.06 and 0.03 mg/mL. Ten microlitres of each standard solution was injected into the HPLC system to construct the standard curve.

The HPLC analysis was carried out on an Agilent 1200 series system equipped with an autosampler, a quaternary pump system, a photodiode array detector and a PL-ELS 2100 Ice detector (Polymer Laboratories, Varian Inc.). Chemstation for LC 3D system software and PL-ELS 2100 Ice Control (Firmware 2.0.2, version 2.0) were used for data handling. The samples were separated with a Phenomenex Luna C_{18}-RP (250 × 4.60 mm, 5.0 µm) column at room temperature. The mobile phase was isocratic for the first 9 min and consisted of water, adjusted to pH 3.0–3.5 with trifluoro acetic acid (TFA) (solvent A) and acetonitrile (solvent B) at a ratio of 30 : 70. The flow-rate was 1.0 mL/min. After the initial 9 min, solvent B (100%) was used for 5 min to clean the column after which the system was returned to 70% solvent B for a further 5 min to re-equilibrate the system. The parameters of the ELSD system differed from the published method (Peng *et al.*, 2006) and were set as to obtain the highest sensitivity. The evaporation temperature was set to 50°C and the nebulisation temperature to 45°C while the nitrogen flow was set at 1.4 L/min.

Table 1. Eight different *A. annua* samples used for the quantitative analysis of ART. Information regarding their origin, plant parts, storage conditions and the year of harvest is also included

Sample number	Country of origin	Plant parts	Storage conditions	Year of harvest
1	South Africa	Leaves/flowers	Poor (not controlled)	1999
2	South Africa	Leaves/flowers	Poor (not controlled)	2002
3	Tanzania	Leaves	Poor (not controlled)	2005
4	South Africa	Leaves	Well (controlled)	2006
5	Tanzania	Leaves	Well (controlled)	2006
6	Cameroon	Leaves	Well (controlled)	2007
7	Germany	Leaves	Well (controlled)	2007
8	Mozambique	Leaves	Well (controlled)	2007

TLC analysis

The methods reported in three recent papers were reproduced in order to determine the quantity of ART (Koobkokkruad *et al.*, 2007; Widmer *et al.*, 2007; Marchand *et al.*, 2008). We found that the method of Widmer *et al.* (2007) was the most suitable for our analytical setup. A standard solution was prepared by dissolving 10.1 mg of ART into 100 mL of toluene (101.0 ng/μL), followed by diluting the stock solution 10 times with toluene (10.1 ng/μL) to cover the linear working range. The standard curve was prepared by applying five different volumes (2, 4, 6, 8 and 10 μL) of the standard solution to obtain five different concentrations. The samples were diluted with acetonitrile so as to fall within the standard curve range. The dipping reagent consisted of 100 mL of ethanol, 80 mL of water, 20 mL of acetic acid, 4 mL of sulfuric acid and 2 mL of anisaldehyde.

Sample application was performed by a Camag Automatic TLC Sampler 4 with winCATS–Planar Chromatography Manager software. Sample volumes of 5 μL were applied as 8 mm bands with the spray-on technique. A twin trough chamber was filled (10 mL) with a mixture of cyclohexane, ethyl acetate and acetic acid (20 : 10 : 1) and presaturated for 20 min. The HPTLC plates were developed in the chamber over a distance of 70 mm from the lower edge of the plate. The developed plates were dried with a hair dryer with cold air for 5 min.

For derivatisation, the developed plates were immersed into the dipping reagent for 1 s after which 1 min was allowed for complete absorption of the reagent, followed by heating the plates at 100°C for 12 min. The quantitative analysis of ART was carried out with a Camag TLC Scanner 3 with winCATS–Planar Chromatography Manager software. Densitometric evaluation was performed in fluorescence mode at 520 nm with cut-off filter at 540 nm using a tungsten lamp. The size of the scanning slit was set at 4.00 × 0.20 mm and the scanning speed at 20 mm/s with a data resolution of 100 μm/step.

LC-MS analysis

A standard solution was prepared by dissolving 10 mg of ART in 1 mL of acetonitrile. This stock solution was diluted 10 times to give standard solution 1 (1 mg/mL). This solution was further diluted 10 times to give standard solution 2 (0.1 mg/mL). The calibration curve was prepared by injecting seven different volumes of standard solutions 1 and 2 to obtain a linear working range. The LC-MS analysis was performed on an Agilent 1100 series LC-MSD equipped with an Agilent Eclipse XDB C_{18} (150 × 4.6 mm, 5.0 μm) column at 25°C (Wang *et al.*, 2005). The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient system employed differed from the reported method (Wang *et al.*, 2005) because of peak overlap with an unknown compound. We therefore had to adapt the mobile phase in order to separate ART from the other components. The mobile phase program consisted of 55% B, isocratically for 9 min, followed by an increase to 100% B in 1 min for 2 min, after which the system was returned to 55% B in 1 min and was kept at 55% for 2 min to re-equilibrate. The total run time per sample was therefore 15 min.

The flow-rate of 1 mL/min employed also differs from the literature (Wang *et al.*, 2005), which used a flow-rate of 1.2 mL/min. Of each sample 5 μL was injected. The LC-MS was equipped with a splitter to split 90% of the flow to the waste and 10% to the MS. This was needed in order to reduce the amount of water entering the MS. The electrospray ionisation mass spectrometer (ESI-MS) was operated under positive ion mode and SIM ion monitoring was used to record the abundance of the $[M + 1]^+$ adduct molecular ion peak at *m/z* 283 for ART. Investigation of the total ion chromatogram indicated that this ion was the most abundant. The ESI was operated by using a capillary voltage of 3.5 kV. High-purity nitrogen was employed as drying gas at a flow-rate of 5 L/min [Wang *et al.* (2005) used 12 L/min], and the drying gas temperature was set at 350°C. Nitrogen gas was also used as the nebuliser gas at 35 psig [Wang *et al.* (2005) used helium gas at 60 psig]. The peak area of the SIM ion was used to calculate the concentration based on the standard curve.

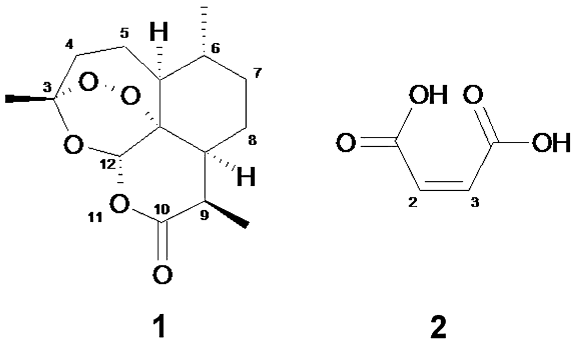

Figure 1. Chemical structures of ART and maleic acid (internal standard for qNMR analysis): 1 = ART, the proton at H-12 was used for quantification; 2 = maleic acid, the protons at H-2 and H-3 were used for ART quantification.

Quantitative NMR parameters

The validated qNMR method of Castilho *et al.* (2008) was used for the quantification of ART. We did, however, make one adjustment to the published method. We did not make use of a standard curve of ART but made use of an internal standard which are not chemically related to ART (maleic acid). Because of molar equivalence of proton signals in NMR spectroscopy there is no need to make use of standard curves in qNMR analysis (Pauli *et al.*, 2005). ^{1}H-NMR spectra were recorded at 25°C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. Each ^{1}H-NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width = 30° (11.3 μs) and relaxation delay = 1.5 s. A pre-saturation sequence was used to suppress any residual water signal with low power selective irradiation at the water frequency during the recycle delay. FIDs were Fourier transformed with line broadening (LB) = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and referenced to the residual CD_3OD at 3.30 ppm, using XWIN NMR (version 3.5, Bruker). For quantification of ART the singlet of H 12 at 6.015 ppm of ART and the singlet of H 2 and H 3 at 6.280 ppm of maleic acid were used (Fig. 1).

Results and Discussion

Artemisinin content

Table 2 gives the quantitative results of the analysis. The results are expressed in mg of ART per gram of dried *A. annua* plant material. According to the WHO, plant material should be dried and extracted as soon as possible as the ART content will decrease within 6–12 months after harvesting, to within a level where the ART cannot be economically extracted. This conclusion is supported by a study performed in China (WHO, 2006). During our tests the oldest sample analysed contained 5.8 mg (based on the qNMR analysis) of ART. This specific sample was 9 years old and had been stored in uncontrolled conditions. The harvest dates of all the samples do not give a clear indication that prolonged storage causes a large decrease in the ART content. The sample with the lowest yield (3.8 mg) was harvested in 2002 while the highest yielding sample was harvested in 2004 (8.9 mg). It therefore appears that the prolonged storage of the plant material does not have a large influence on the levels of ART, as was previously thought.

The quantification of ART in the plant samples yielded unexpected results. We tested validated published methods in order to compare the different techniques. Our reasoning behind this is that scientists who are working with *A. annua* will use these

Table 2. The quantity of ART in the plant samples expressed in mg of ART/ gram of dry *A. annua* plant material

Sample	LC-ELSD	TLC	LC-MS	qNMR
1	5.1 ± 0.4	5.3 ± 0.3	4.8 ± 0.2	5.8 ± 0.3
2	3.3 ± 0.2	3.4 ± 0.2	3.3 ± 0.5	3.8 ± 0.1
3	5.9 ± 0.1	8.8 ± 0.7	5.2 ± 0.1	5.6 ± 0.1
4	9.3 ± 0.1	10.9 ± 0.6	8.7 ± 0.9	8.9 ± 0.3
5	6.4 ± 0.1	8.3 ± 0.6	6.0 ± 0.4	6.6 ± 0.1
6	6.2 ± 0.2	8.0 ± 0.3	6.1 ± 0.2	5.5 ± 0.2
7	7.9 ± 0.2	9.6 ± 0.1	7.4 ± 0.3	7.8 ± 0.3
8	5.5 ± 0.1	8.0 ± 0.4	5.0 ± 0.7	5.8 ± 0.3
Estimation range[a]	−0.7 to +0.7 mg	−0.5 to +3.2 mg	−1.0 to +0.6 mg	—

[a] Note: the estimation range is expressed as the sample with the largest underestimation (− value) to the sample with the largest overestimation (+ value) compared to the qNMR result.

methods as published, especially when a large number of publications confirm the use of a specific technique. Each of the techniques used was also looked at for ease of use, and the problems associated with each technique were noted. We also chose an extraction method which reported a >95% recovery of ART (Peng *et al.*, 2006). We used the same samples for all of our analysis, excluding the qNMR analysis, for which we extracted separate samples. We found that the reproducibility of the extraction method was high, based on the standard deviations observed for the crude extract weights (data not included), and the determined ART concentration of all the samples tested.

From the results it can be seen that the standard deviations indicated that the precision of the analysis on all the detectors was satisfactory. The results from the HPLC-ELSD detection system corresponded closely to the result obtained from qNMR. The estimation range for the ESLD for all samples was ±0.7 mg. The results obtained from the LC-MS method had a slightly bigger estimation range of between −1.0 and +0.6 mg. The TLC results indicated a relatively large estimation range of between −0.5 and +3.2 mg.

Existing problems of the analytical methods and possible solutions

Because of the advantages of qNMR (discussed in the Introduction) we decided to compare all the results to the qNMR result. This is, however, an arbitrary choice. The HPLC-ELSD method gave the most comparable results to the qNMR method. There were, however, certain problems with the use of this detector. Among all the published HPLC-ELSD methods, one group made use of a linear fit to produce their standard curve (Peng *et al.*, 2006). We could not reproduce this, although we attempted to achieve this by changing various settings on the detector to influence the response. The quadratic fit did however give us the required regression value of >0.98. Interestingly the shape of the peak appeared to have a large influence on the integral value, e.g. sharp and symmetrical peaks gave an arbitrary value of 100 while slightly asymmetric peaks gave lower values of about 80. To investigate this phenomenon, we tested various columns and compared the integral value and the influence of the peak shape on this value with the UV-absorbing tetrahydrocannabinol on a UV and ELSD system (as ART does not absorb UV we used tetrahydrocannabinol). While the UV detector gave similar peak areas, no matter what the peak shape was, the peak shape largely influenced the integral value obtained from the ELSD system. When the column was removed and the sample injected, the resulting sharp peak gave an integral value of 100, while with a column it would give an integral value of 60. The reason for this is that the ELSD is not a linear detector. If a compound reaching the detector spreads out over time it will give a lower response with a lengthening of time. The shorter the time the higher the response will be. Therefore if a large number of samples are injected with a subsequent degradation of the column efficiency and peak shape (e.g. tailing), the ELSD detector might start to underestimate the amount of ART. This might not be a serious problem if standards are injected at regular intervals. Another potential problem with the ELSD detector is that the response is largely influenced by the flow-rates of the mobile phase and the gas. Slight variations in the flow-rate can cause large fluctuations (caused by the quadratic response) in the obtained results. In order to minimise this potential problem, it is recommended that the flow-rates (and the pump backpressure) should be monitored during the analysis of samples. Most modern HPLC software can perform this task. Visual inspection will indicate if any problems occurred during the analysis of the samples.

We tested three published TLC methods (Koobkokkruad *et al.*, 2007; Widmer *et al.*, 2007; Marchand *et al.*, 2008), and came to the conclusion that it is a rather difficult method to use, with the potential of a relatively large variation in the results. In chromatography, peak overlap will always remain a problem and the possibility that peak overlap occurs is relatively large in these crowded chromatograms. The main problem associated with this technique was the number of steps involved, and especially the derivatisation step (the more steps needed the higher the chance for systematic and random error). This step must be carefully monitored in order to obtain reproducible results. The results furthermore suggested that the TLC method underestimated at low concentrations and overestimated at higher concentrations (Table 2). The average overestimation of all the samples was about 25% compared with the qNMR results. The main advantages of the TLC method are that it is relatively inexpensive compared with the other methods and that multiple samples can be analysed at the same time.

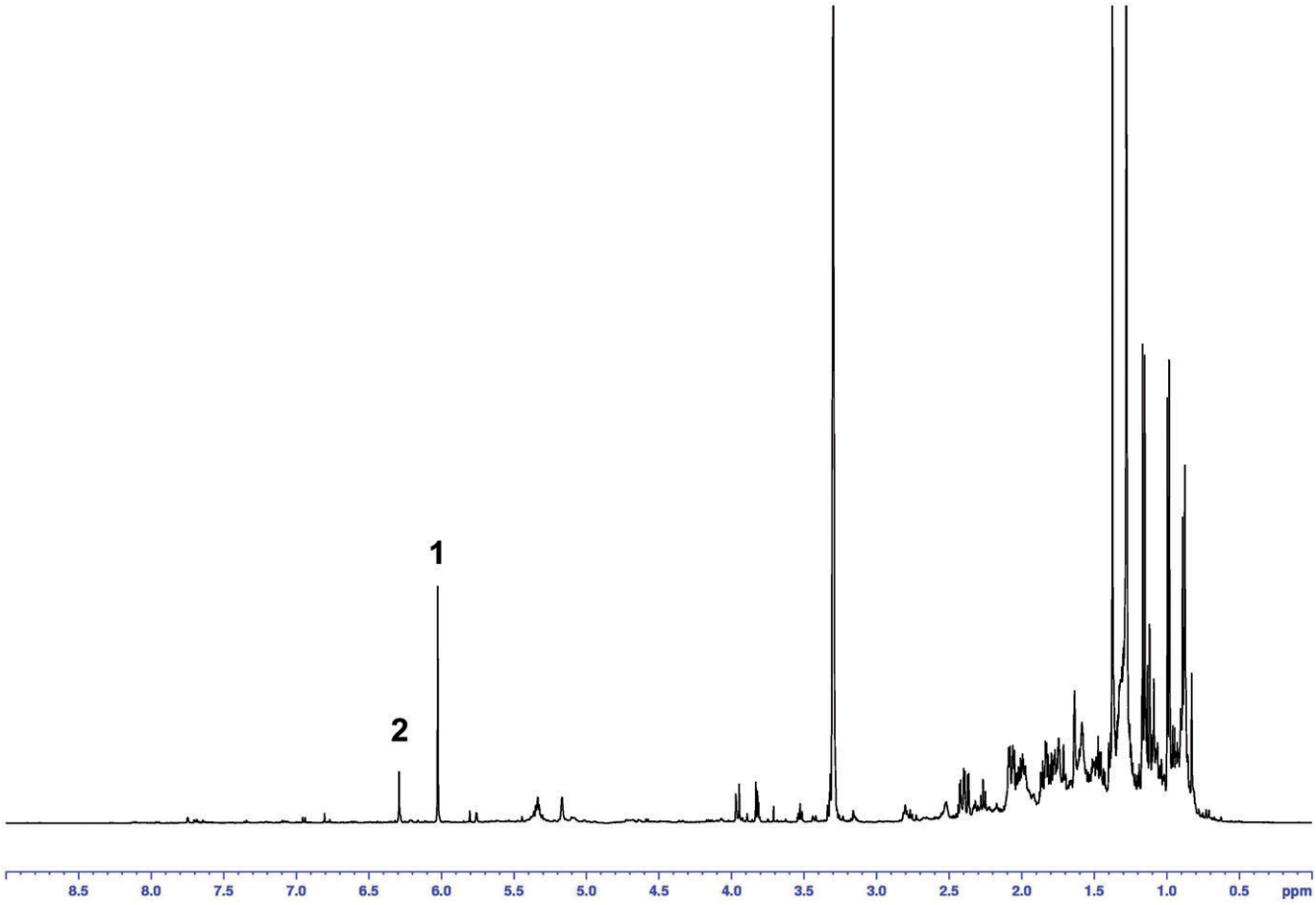

Figure 2. Typical ^{1}H-NMR spectrum of an *A. annua* extract: 1 = the singlet from H-12 of ART at 6.015 ppm used for quantitation and 2 = the singlet from two symmetric protons at H-2 and H-3 of maleic acid at 6.280 ppm.

The LC-MS method is relatively easy to set up and simple to use. The main problem associated with the LC-MS was the accumulation of contaminants in the spray chamber. This could clearly be seen in a gradual increase of the noise level in consecutive chromatograms. To solve this problem the spray chamber had to be cleaned regularly and the samples had to be injected in reverse order. Therefore, all the samples had to be analysed more than once, with the first set of analysis starting with sample 1 and the second set of analysis starting with sample 8. This sequence of analysis was repeated on three consecutive days. When all results were compared it was clear that, after a certain number of injections of a specific sample, the LC-MS started to underestimate the ART content depending on where in the sequence the sample was. The level of underestimation remained relatively small, e.g. >5%. The results indicated an underestimation of about 6% which can partly be explained by the increase of the noise level due to the accumulation of contaminants in the spray chamber. In addition, due to the fact that ion suppression (or enhancement) and the matrix effect can also lead to under- and over-estimation (Van Nieuwerburgh *et al.*, 2006), these effects should be carefully investigated by studying the fragmentation patterns of the standards and the samples and by performing spiking experiments for accurate and precise quantitation.

A qNMR method reported recently was adapted in order to develop our own qNMR method (Castilho *et al.*, 2008). The authors of this method made use of standard curves to quantify ART. This method can be used but it is not necessary to include a standard curve to quantify the target compound. Because NMR signals are molar equivalent, no standard curve is required. With the addition of an internal standard the concentration can easily be calculated (Kim *et al.*, 2003; Choi *et al.*, 2004). The qNMR method appeared to be accurate (corresponds well with LC-MS and LC-ELSD) and quite easy to perform. In addition it does not suffer from reproducibility problems associated with chromatographic techniques. The only requirements for qNMR is that the peak to be integrated should be stable (e.g. no OH protons) and that it should not overlap with any other peaks. Figure 1 shows the chemical structure of ART and the internal standard, maleic acid, with the protons used for quantification. In the case of ART, the peak at 6.015 ppm is the ideal stable proton signal and was therefore also used for integration (Fig. 2). Because of the insensitivity of NMR compared with other detectors, this method will however not be suitable for detection of small quantities of ART, as are usually encountered in pharmacokinetic experiments. It is, however, ideal for quantifying ART at higher concentrations as is found in the *A. annua* plant material.

Conclusions that can be drawn from the results are that it appears that *A. annua* can be stored for longer periods of time without a large degree of degradation of ART. The WHO also recommends that, when the plant material is stored for longer than a 6 month period, the concentration of ART should first be tested, before extraction. The accurate quantification of ART remains a problem based on the relatively large variation in the results obtained from the different detection methods. There is

almost a 30% difference in the quantity of ART based on the TLC and LC-MS results. By excluding the TLC results, the remaining three quantification methods by and large confirm the accuracy of these analytical techniques. This also proves that qNMR can be used to quantify ART in the plant material without the need for a calibration curve. In addition, qNMR can also be employed without the use of an ART standard (Pauli, 2001; Rizzo, Pinciroli, 2005). This direct quantification method with the least amount of steps involved should therefore form the basis for the quantification of ART in the plant material. The reported analysis time of about 10 min per sample can also be reduced to less than 3 min per sample. This can be achieved by reducing the number of scans, making use of a higher field magnet and/or make use of a cryoprobe and thereby improve the sensitivity by reducing the noise levels. The accurate quantification of ART should be further investigated by means of an inter-laboratory study focusing on developing a GLP quantitation method for ART. In conclusion, direct quantification methods with simple sample preparation can be seen as more accurate than indirect approaches, because more steps increase the chance for systematic and random error. While small standard deviations suggest high reproducibility and precision, this might indicate that the direct quantitative approach, qNMR, might be more accurate than the indirect quantitative methods such as LC-MS, HPLC-ELSD and TLC.

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