doi:10.2533/chimia.2010.755

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Quantification of the Total Amount of Artemisinin in Leaf Samples by Thin Layer Chromatography

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Abstract: Artemisinin is a natural molecule highly active against malaria. At present, the extraction of this molecule from the leaves of *Artemisia annua* L. remains the only viable method to produce cheaply large quantities of artemisinin. Agronomic research on this plant species aims to improve agricultural yields, to decrease production costs and to ensure a steady global supply of artemisinin. These research activities require an easy, rapid, low cost, and reliable analytical technique to quantify the artemisinin content in the leaves. Thin layer chromatography (TLC) methods to quantify this molecule have already been published. However, this method does not allow the quantification of the total artemisinin content in the leaves. In order to validate the TLC method, results obtained with this method were related to results for the same samples obtained by accelerated solvent extraction and high pressure liquid chromatography with an evaporative light scattering detector (ASE-HPLC-ELSD). Using the Nernst partition law, a corrective factor of 1.21 is suggested to enable information about the true total amount of artemisinin in leaf samples to be obtained within a range of 0.25 to 3%. In conclusion, this study proposes for the first time a corrective factor in order to quantify the total artemisinin content of *A. annua* leaves with TLC.

Keywords: Artemisinin · Recovery rate · TLC · Total quantification method

Introduction

Artemisinin (Fig. 1) and its derivatives[1] are drugs used to treat malaria. Recommended by the World Health Organisation (WHO), they are used in combination with other compounds as bi- or polytherapies. These treatments are used against strains of *Plasmodium falciparum* Welch that are resistant to traditional antimalarial treatments.[2,3] Artemisinin is a sesquiterpene lactone with an endoperoxide bridge difficult to synthesize at low cost, so the direct extraction from the leaves of *Artemisia annua* L. remains a necessary step for the drug manufacture.[4,5] Agronomical research is active on this species, with the principal aim to improve the yield in artemisinin, in order to decrease the quantity of plants to be treated during extraction procedures.[6–8] Mediplant has been working for more than 20 years on this *Asteraceae* and has acquired thorough knowledge on cultural techniques, drying and stocking procedures. Mediplant has also developed hybrids that are particularly rich in artemisinin.[9] This kind of research requires an easy, rapid, low cost, and reliable analytical technique to quantify the artemisinin content of numerous types of samples. Knowledge of the exact content is also important information for the producers as well as for industrial companies. They will thus know the quality of the plant they are buying or selling, the quantity needed to produce a certain amount of artemisinin and the efficiency of their extraction technique. Quantification of artemisinin can be carried out easily by thin layer chromatography (TLC).[10,11] However, most extraction procedures do not allow the complete extraction of the active substance of a sample. This fact implies that the quantification is only partial. However, the obtained results can be corrected if the total quantity of the active substance is known. Total quantification can be attained by several consecutive extractions and extrapolation using the Nernst partition law.

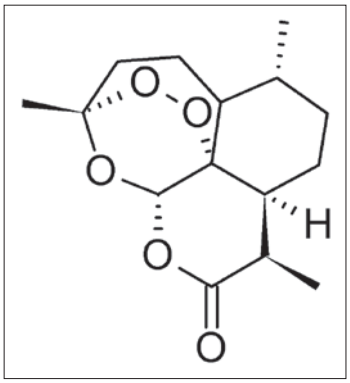

Fig. 1. Structure of artemisinin.

This article confirms the validity of the TLC quantification method in plants that contain between 0.5 and more than 1.5% of artemisinin and proposes a corrective factor based on the total quantification of this molecule in the plant.

Experimental

Thin Layer Chromatography

A stock solution of artemisinin (provided by Botanical Extracts EPZ Ltd, batch n° 127/1/2901, NMR purity: >99.0%) was prepared in toluene (1 mg/ml). Five standard solutions were then prepared from this stock solution and deposed on a silica plate. Dilutions were carried out with toluene to 100 ng/µl, 150 ng/µl, 200 ng/µl, 250 ng/µl and 300 ng/µl.

Artemisia annua L. plants were grown on the experimental site in Conthey, Switzerland. They came from a genotype collection maintained *in vitro*. Plants were harvested in autumn, before freezing occurs, and dried at 35°C with pulsed air. Leaves were separated from the stems, and then ground with a centrifuge grinder (≤0.5 mm). 100 mg of powder were mixed with 5 ml of toluene in a Polytron type PT 20.00 (Kinematica GmbH, Lucerne, CH) for 30 seconds, and then centrifuged at 3770 rpm for 5 minutes. The extract was applied immediately on the plate.

In order to avoid bias due to differences in water content of dry leaves, dry matter content was determined. Results are expressed in percent of artemisinin in the dry matter using the formula: % of artemisinin / % dry matter * 100 = Result in %. The moisture content was determined for each sample by weighing exactly 3 g powder. This sample was dried at 105 °C for 12 h, then cooled in a desiccator and weighed again.

One microliter of plant extract was applied at 15 mm of the inferior edge of precoated silica gel TLC plates 60F254 (Merck, Darmstadt, D), 10 × 20 cm. The manual application was done with

Table 1. Sequence of deposit on silica plate, position 1 to 15, sample type and artemisinin content.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Standard	Known sample	Sample 1	Sample 2	Sample 3	Standard	Standard	Standard	Standard	Standard	Sample 2	Sample 4	Sample 5	Sample 6	Standard
100 ng					100 ng	150 ng	200 ng	250 ng	300 ng					100 ng

a microcapillary of 1 microliter, using a Nanomat (Camag, Muttenz, CH). Fifteen samples were applied on one plate. Samples were applied as indicated in Table 1. To avoid edge effects, a standard solution of 100 ng of artemisinin was applied at positions 1 and 15. One known sample was applied at the second position; this sample was analyzed previously by HPLC, this enabled us to check the quality of the plate. The second unknown sample (at the fourth position) was applied again at the eleventh position in order to determine the within run precision. A TLC plate was considered unusable when the obtained value of the known sample differed too much from the expected value. Plates were also discarded when the standard deviation was greater than 10%.

Chromatography was performed on the silica plate with n-hexane and diethyl ether (6:5, v/v) as the eluent in a twin trough developing chamber (Camag); the migration distance is 60 mm (about 6 min migration time). Pre-equilibration with a saturation pad (Camag) took 30 min. The migration was ascendant and linear. For thermal *in situ* derivatization of artemisinin spots, the developed plate was dried for 3 min, immersed for 1 s in a solution of acetic acid, sulfuric acid and anisaldehyde (100:2:1, v/v/v), and then heated on a TLC plate heater III (Camag) at 105 °C for about 5 min. Artemisinin appeared as a pink spot with an R_f value of 0.20.

Densitometric evaluation was performed by TLC Scanner 2 (Camag) by measuring absorbance at 530 nm (tungsten lamp, scanning speed 4 mm/s). The calibration range was 100–300 ng/µl of artemisinin. Statistical data were obtained with CATS software (Camag).

Validation Method

The TLC method was validated by a total quantification method. Although high performance liquid chromatography with ultraviolet detection (HPLC-UV) is widely available, it has not been used for artemisinin analysis because of the lack of UV absorption reported for this lactone.[12] The total quantification was done by extraction with an accelerated solvent extractor ASE 200 (Dionex Corp., Sunnyvale, (CA) USA), followed by separation and quantification with a series 200 HPLC system (Perkin Elmer, Waltham, (MA) USA) using an evaporative light scattering detector Sedex LT-ELSD 75 (Sedere, Alfortville, F). Overall, the HPLC-ELSD method appears to be the most robust method for routine quantification of artemisinin in plant extracts, either for the purpose of quantification of artemisinin content in the leaf, or for the optimization of extraction/purification protocols. The HPLC-UV method is not recommended for the analysis of extracts, but only for the analysis of the purity of bulk artemisinin.[13] A 3 g sample of ground plant material and 7 g of bulk isolute sorbent were introduced in a 33 ml extraction cell which was placed in the ASE 200. Three replications were done for each plant sample. The temperature was set at 40 °C and the pressure at 100 bar. Three consecutive extractions with an ethanol-water solution (3:1, m/v) were performed on the same cell. 10 µl of these three extracts were eluted with a 0.6 ml/min flow on a Nucleosil 100-3 C18 HD column 125 × 3 mm i.d. (Macherey-Nagel, Düren, D) using an acetonitrile-water (3:2, v/v) eluent. The conditions of the ELS detector were: gas pressure: 2.4 bar, nebulisation chamber and heating chamber temperatures: 40 °C. A matrix effect test was done by adding a known amount of artemisinin (same amount compared to the initial content of artemisinin in the plant) to the 33 ml extraction cell. The extraction was done in the same way as described above. This method allows for a correction of the TLC results.

Results and Discussion

Thin Layer Chromatographic Conditions

With the TLC method, six different samples on one plate could be analyzed. The method described here is adapted from Gaudin and Simonnet.[11] This new method allows the determination of the quantity of artemisinin contained in new hybrids. These new selections contain larger amounts of the active principle than earlier selections. The concentration of artemisinin in different samples is measured using the calibration line of the five spots of artemisinin standard scale. This calibration line is calculated by the Camag Wincats software. The linearity of the standard scale must be checked. The calibration line is linear between 100 and 300 ng, the correlation coefficient is 0.99 (Fig. 2). The scale and the method used allow a direct measure of the percentage of artemisinin in the sample. 100 ng correspond to 0.5% artemisinin in the dry ground sample, 150 ng to 0.75%, 200 ng to 1.00%, 250 ng to 1.25% and 300 ng correspond to 1.5% of active principle in the powder.

The quantification limit is 0.45%; if a sample contains less or more active principle, it is always possible to apply less or more plant extract (0.5 ml to 2 ml). It is thus possible to quantify extracts containing between 0.25% and 3%. The detection limit has not been determined.

We analyzed different leaf samples that contain from 0.5 to more than 1.5% artemisinin (Table 2). Four repetitions were done for each sample. The mean of the standard deviations is 0.07. The samples contain between 0.49% and 1.3% artemisinin. The mean content of dry matter in the samples is 91.4%.

Total Quantification by HPLC-ELSD

For any extraction technique, it is impossible to extract the total content of artemisinin; therefore the final result (Table 2) has been

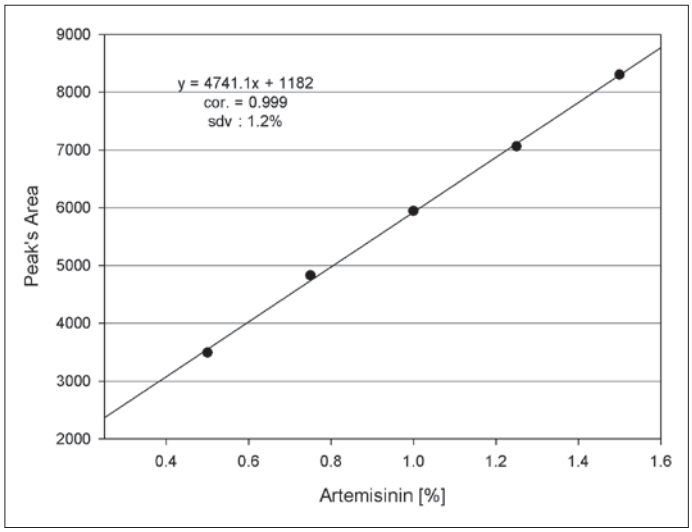

Fig. 2. TLC Calibration curve for artemisinin.

Table. 2. Artemisinin content of seven genotypes of *A. annua* grown in Switzerland in 2007; results are expressed on the dry matter.

	% of artemisinin		
Sample	HPLC-ELSD	TLC	TLC recovery rate
4 I	0.71	0.50	70%
B 38	0.63	0.49	78%
C 48	1.06	0.84	79%
C 27	1.13	0.86	76%
C 50	1.02	0.88	86%
P 49	1.53	1.26	82%
5 III	1.69	1.32	78%

extrapolated using Nernst partition law. Three consecutive extractions on the same ASE cell suffice to extract a maximum percentage of artemisinin (between 86 and 97% depending on samples). Three replications were done for each plant sample (Fig. 3). The advantage of the HPLC-ELSD measurement is that it avoids the risk of some derivatization methods that the derivatization is incomplete. The ELSD detection allows a 60 ppm detection limit.

Tests performed to determine a possible matrix effect yielded recovery rates of 100.8 ± 0.3%. This shows that the quantification of artemisinin in extracts of *A. annua* is not influenced by the presence of other components, that there are no biases and that the error is acceptable because it is less than 1% (0.8%). These experiments show that the artemisinin standard does not have the same solubility as the artemisinin present in the plant because the standard has a crystal form different from that in the plant.

Because of the excellent correlation between both analytical methods the recovery rate (Table 2) and a corrective factor (Fig. 4) can be calculated. The artemisinin recovery rate for the TLC method is 78%, the standard deviation is 0.05. With most extraction procedures a complete recovery of the active principle cannot be realized. The presented method for extraction and TLC quantifies only a part of the artemisinin contained in the plant. This is probably due to the extraction procedure. A fraction stays behind in the vegetal matrix. It is also known that during centrifugation, up to 20% of the substance can stay behind in the pellet under the effect of the centrifugal force. Gaudin and Simonnet[11] obtained a recovery rate of 91% with the same extraction procedure. This value shows that a small loss occurs during centrifugation. It is also known that the extraction solvent can be completely saturated with a mixture of molecules that possess similar affinities; these molecules will hinder the solubilization of artemisinin in the solvent. The selectivity of toluene has not been tested.

A corrective factor can be applied to the TLC results obtained with this method, in order to know the exact quantity of artemisinin contained in an extract. This corrective factor is 1.21. It means that based on the TLC results, it is possible to know exactly the total quantity contained in the sample, using a simple calculation.

Conclusions

TLC is a rapid, low cost and reliable analytical technique for quantifying the artemisinin content of numerous types of samples. The selectivity of the extraction and derivatization procedures is good. Almost no other compounds appear on the plate. The determined corrective factor permits the true quality of harvested plants to be evaluated, which is useful for selecting elite plants as well as to evaluate the quality of plants used in the extraction procedures.

Acknowledgements

The authors thank Botanical Extracts EPZ Ltd, Kenya, for the kind supply of artemisinin standard. They also thank Dr. Frans Zonnevijlle for proof reading the article.

Received: August 20, 2010

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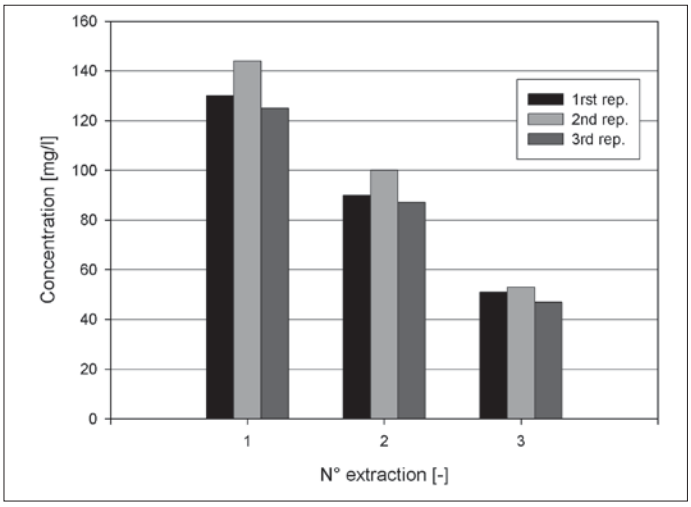

Fig. 3. Artemisinin content of three consecutive extractions with the ASE. Three repetitions were done for each sample.

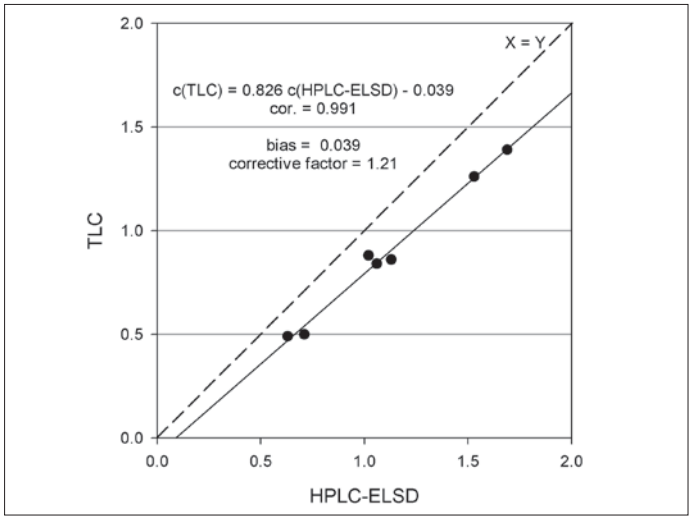

Fig. 4. Correlation of artemisinin content analyzed by two different methods, corrective factor.