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Quantification of Artemisinin in *Artemisia annua* Extracts by ^{1}H-NMR

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Abstract: Artemisinin is a polycyclic sesquiterpene lactone that is highly effective against multidrug-resistant strains of *Plasmodium falciparum*, the etiological agent of the most severe form of malaria. Determination of artemisinin in the source plant, *Artemisia annua*, is a challenging problem since the compound is present in very low concentrations, is thermolabile and unstable, and lacks chromophoric or fluorophoric groups. The ain of this study was to develop a simple protocol for the quantification of artemisinin in a plant extract using an ^{1}H-NMR method. Samples were prepared by extraction of leaf material with acetone, treatment with activated charcoal to remove chlorophylls and removal of solvent. ^{1}H-NMR spectra were measured on samples dissolved in deuterochloroform with *tert*-butanol as internal standard. Quantification was carried out using the δ 5.864 signal of artemisinin and the δ 1.276 signal of *tert*-butanol. The method was optimised and fully validated against a reference standard of artemisinin. The results were compared with those obtained from the same samples quantified using an HPLC-refractive index (RI) method. The ^{1}H-NMR method gave a linear response for artemisinin within the range 9.85–97.99 mM (r^2 = 0.9968). Using the described method, yields of artemisinin in the range 0.77–1.06% were obtained from leaves of the *A. annua* hybrid CPQBA × POP, and these values were in agreement with those obtained using an HPLC-RI. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: NMR; quantification; artemisinin; *Artemisia annua*.

INTRODUCTION

The incidence of malaria, one of the most common diseases in the tropical world, is increasing with no clear solution in view. It is responsible for more than one million deaths worldwide each year (WHO, 2005) and it threatens about 40% of the world's population, causing mortality, morbidity and socio-economic loss. New research suggests that malaria may be helping to spread the AIDS virus across Africa (Abu-Raddad *et al.*, 2006).

Malaria has been known since antiquity as a disease spread by the female of the *Anopheles* mosquito. Nearly all cases of death by malaria are caused by the parasite *Plasmodium falciparum* (Guerin *et al.*, 2002). Widespread resistance of *P. falciparum* to classical anti-malarial agents such as chloroquine, mefloquine and antifolates has made the disease situation even more difficult to manage in endemic malaria areas. The interest in plants as potential sources of new anti-malarial drugs has been stimulated since the isolation and clinical use of artemisinin (**1**; Fig. 1) from a Chinese plant, *Artemisia annua* (Asteraceae), that has been used for many centuries in Chinese folk medicine for the treatment of fever and malaria and is the only natural source of artemisinin.

Artemisinin is a polycyclic sesquiterpene lactone bearing an endoperoxide bridge that probably induces the anti-malarial activity (Klayman, 1985; El-Sohly *et al.*, 1990; Price *et al.*, 1999; Robert *et al.*, 2002). The most important derivatives of artemisinin are artemether (**2**), arteether (**3**), artesunate (**4**) and dihydroartemisinin (**5**) (Dhingra *et al.*, 2000), whilst its main precursors are artemisinic acid (**6**) and dihydroartemisinic acid (**7**) (Brown, 2003; Fig. 1).

Artemisinin and its derivatives are effective against multidrug-resistant *P. falciparum* strains, especially in artemisinin combination therapies (ACT), and have been in use in Southeast Asia and in Africa without any reported cases of resistance (Mutabingwa, 2005). In 2005, the estimated global demand for anti-malarial drugs was 120 million adult treatment courses (WHO, 2005), thus approximately 114 tons of artemisinin are required annually (Olliaro and Taylor, 2004). Although the total synthesis of artemisinin has been achieved (Schmid and Hofheinz, 1983), it is not competitive in price with the natural product. On the other hand, *A. annua* is easy to cultivate (WHO, 2006), and researchers have been able to produce hybrids rich (up to 1.5%) in artemisinin (Magalhães *et al.*, 2004).

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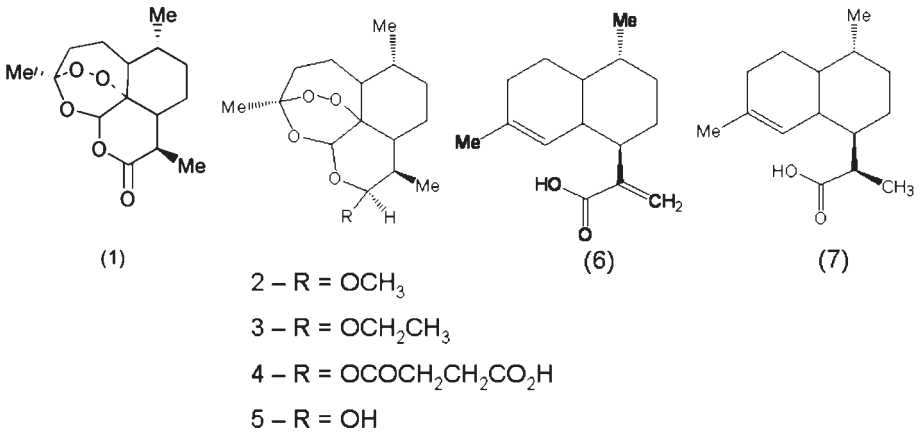

Figure 1 Structures of artemisinin (**1**), artemether (**2**), arteether (**3**), artesunate (**4**), dihydroartemisinin (**5**), artemisinic acid (**6**) and dihydroartemisinic acid (**7**).

In order to allow artemisinin to be cheaply available in developing countries, there is a need to find an efficient method of extracting artemisinin from dry leaves that is simple enough to be performed *in situ*. Lapkin *et al.* (2006) published a review concerning several established and emerging technologies for artemisinin extraction. Liquid solvent extraction, employing toluene, *n*-hexane, chloroform or petroleum ether, is currently the most applied technique to obtain artemisinin (Christen and Veuthey, 2001).

In the last decade, new extraction techniques have been developed. Kohler *et al.* (1997) reported the extraction of artemisinin by supercritical fluid extraction and analysis by supercritical fluid chromatography. Extraction of artemisinin by microwave-assisted extraction (Kohler, 1999) was carried out using water (although this was not successful since the plant extract degraded in this solvent), ethanol, toluene or *n*-hexane. Artemisinin recovery was similar with ethanol, toluene and *n*-hexane, although the highest selectivity has been obtained with *n*-hexane.

Analysis of artemisinin is a challenging problem since the compound is present in low concentrations in the plant, it is thermolabile, unstable and acid-sensitive and lacks chromophoric or fluorophoric groups; moreover, the intact molecule stains poorly and other compounds in the crude plant extracts can interfere with the detection. Christen *et al.* (2001) have published an excellent overview of the various conventional and unconventional methods that have been proposed and assessed in order to detect and quantify artemisinin.

TLC is not a reliable technique to quantify artemisinin owing to the poor staining characteristics of the intact molecule and interference from other constituents of the plant. Artemisinin is a thermolabile compound (stable up to 150°C, but degrades into several products when heated at 180–200°C) and cannot be determined without degradation by GC (Dhingra *et al.*, 2000). Thus, GC methods are based on the linear relationship obtained between the concentration of artemisinin and the peak areas for either of the two thermally degraded products. Reproducibility of the method is rather dubious. HPLC, with UV monitoring, is often used in the analysis of artemisinin and its analogues but it requires a derivatisation procedure (Van Geldre *et al.*, 1997). The endoperoxide bridge in artemisinin and its derivatives allows the electrochemical reduction of the peroxide function. HPLC with electrochemical detection (EC) can be used for artemisinin detection (Charles *et al.*, 1990), and this method is highly sensitive (Dhingra *et al.*, 2000), although it demands the rigorous deoxygenation of all reagents and the maintenance of oxygen-free analysis conditions. Wang *et al.* (2005) developed and validated an HPLC-MS method with selected ion monitoring (SIM) for the analysis and standardisation of artemisinin, and recently HPLC-MS (TOF) was used for the quantification of artemisinin and its bio-precursors quantification (Van Nieuwerburgh *et al.*, 2006). Redher *et al.* (2002) developed a quantification method using HPLC with differential refractometer detection: this HPLC-refractive index (RI) method provided an average recovery of >95% and a limit of quantification of 0.21%.

Radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) offer greater sensitivity and higher specificity than most conventional methods. In RIA, however, radioactive compounds are used and this presents a series of problems including special acquisition and use requirements, uncertain stability, high cost, health hazards and disposal difficulties. ELISA is at least as sensitive as RIA and is safer, and can be a faster and more sensitive method for the detection and quantification of artemisinin (Ferreira and Janick, 1996). In 1993, Jaziri *et al.* reported the establishment of an indirect ELISA method based on the peroxide bridge for antibody specificity to detect artemisinin and closely related compounds in crude

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extracts of *A. annua*. Chen *et al.* (2002) developed a novel, rapid and simple capillary electrophoresis (CE) method for the determination of artemisinin by on-line treatment with 0.20 M sodium hydroxide solution for 3 min at 40°C, where artemisinin was automatically and reproducibly converted to the strongly UV-absorbing compound, Q292 (Chen *et al.*, 2002). Analysis was carried out in less than 12 min after conversion of artemisinin in a flow injection system that was coupled to CE equipment via a split-flow interface cell.

In the present work, we propose two innovations: the use of acetone as the extraction solvent and the use of ^{1}H-NMR as the technique to quantify artemisinin directly in the acetone extract. The results obtained for the quantification of artemisinin were compared with those obtained for the same extracts submitted to HPLC-RI quantification.

MATERIALS AND METHODS

Plant material. Leaves of *A. annua* were obtained from seeds of an artemisinin-rich hybrid (CPQBA × POP) kindly provided by Professor Pedro Magalhães (UNICAMP, Campinas-SP, Brazil) and cultivated in Madeira, Portugal, during 2003 in the grounds of the Madeira Botanical Gardens. In September, before flowering, the whole plants, about 3.5 m tall, were cut and then dried for a week under direct sunlight. Leaves were separated from stalks and finely ground. Five different samples were used in this study.

Sample preparation. Preliminary experimental data (not presented) indicated that, in order to obtain the highest yields of artemisinin, leaf material should be extracted with commercial acetone (96%) for 14 h under magnetic stirring. Chlorophylls were eliminated from the acetone solution by addition of activated charcoal, and the solvent was removed under reduced pressure at 35°C. This simple process yielded the acetone extracts that were used throughout the study for the quantification of artemisinin.

Chemicals. Artemisinin standard (98% pure) was purchased from Aldrich (Steinheim, Germany) and standards of *tert*-butanol (>98% pure) and camphor (98% pure) were purchased from Merck (Darmstadt, Germany). All chemicals were used as supplied.

Qualitative and quantitative NMR analysis. The unequivocal assignment of the ^{1}H- and ^{13}C-NMR resonances of each compound under study was achieved using a combination of one- and two-dimensional techniques such as homodecoupling, COSY, HMBC, HMQC. NMR spectra were measured using a Bruker (Wissembourg, France) AMX-300 spectrometer operating at 300.135 MHz for ^{1}H and 75.477 MHz for ^{13}C and equipped with a 5 mm QNP probe. The spectra were recorded in deuterochloroform with all shift values (δ) referred to the internal standard tetramethylsilane (TMS) and expressed in ppm.

For NMR quantification, the typical acquisition parameters employed in proton NMR experiments were as follows: pulse width 8.00 μs (flip angle 90°); acquisition time 3.867 s; relaxation delay d_1 3 s for a 64K data table with a spectral width (SW) of 2564 Hz (8.5 ppm); temperature 298K. NMR processing for all samples included phase correction (performed manually for each replicate) and manual baseline correction over the entire spectral range. Peak integration was performed automatically using the NMR spectrometer software.

Calibration. Stock solutions were prepared containing 27.6 mg/mL of artemisinin and 15.35 mg/mL of camphor in a solution of 6.52 mg/mL of *tert*-butanol in deuterochloroform. From these stock solutions, five diluted solutions were prepared in order to obtain calibration curves for each compound.

Standard mixture of artemisinin and camphor. Artemisinin (4.2 mg) and camphor (0.1 mL of 130 mM solution in deuterochloroform) were diluted with 0.4 mL deuterochloroform using *tert*-butanol (6.52 mg/mL) as internal standard. The concentration of each component was thus 29.46 mM for artemisinin and 26.08 mM for camphor.

***Extracts of* A. annua.** The spectra of the extracts were recorded after diluting 100 mg of extract in 1 mL of deuterochloroform containing 6.16 mg of *tert*-butanol as internal standard. For each extract, three replicate samples were analysed.

Quantitative HPLC-RI analysis. Samples of extracts obtained as described above were analysed by HPLC using a Waters (Milford, MA, USA) model 515 chromatographic system with a model 2414 differential refractometer detector operating at 35°C. An Agilent Technologies (Palo Alto, CA, USA) CN100A column (250 × 4.6 mm i.d.; 5 μm) was employed and the mobile phase was water:methanol (60:40 v/v) at a flow rate of 1 mL/min. A calibration curve was constructed using solutions containing between 90 and 900 mg/mL of pure artemisinin, and quantification of the analyte in the extracts was determined from the equation $Y = 1.32 \times 10^{-3}x + 9.57 \times 10^{-4}$, $R^2 = 0.9900$. The analyses were performed by Dr. Mary Ann Foglio and co-workers at CPQBA, UNICAMP, Campinas-SP, Brazil.

RESULTS AND DISCUSSION

A raw acetone extract was analysed by ^{13}C-NMR (data not shown) and ^{1}H-NMR (Fig. 2) in order to identify the

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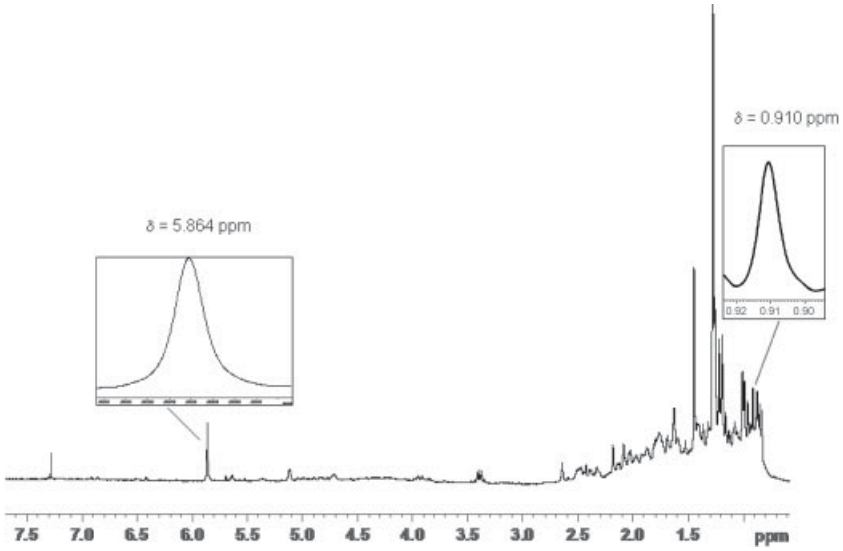

Figure 2 ^{1}H-NMR spectrum of the raw acetone extract of *Artemisia annua*. The signals chosen for the quantitative determination of artemisinin (δ 5.864) and camphor (δ 0.910) are highlighted.

major components. This was achieved by comparing chemical shifts with those of pure standards for artemisinin and camphor, and with published data for dihydroartemisinic acid (Wallaart *et al.*, 1999).

The ^{13}C-NMR spectrum obtained for the standard mixture permitted the identification of artemisinin (all 15 carbon signals; Blasko *et al.*, 1988), camphor (nine out of 10 carbon signals) and dihydroartemisinic acid (13 out of 15 carbon signals; Wallaart *et al.*, 1999). As major components, artemisinin and camphor were chosen to be quantified by ^{1}H-NMR. Although artemisinin was our target analyte, the simultaneous quantification of camphor was performed in order to reinforce the validation of the method.

^{1}H-NMR analysis of the whole extract allowed the choice of signal, or signals, to be used in the quantification procedure and, consequently, the choice of internal standard. For each compound, we chose several signals in order to determine which would be most suitable for ^{1}H-NMR quantification. The final choice of signals is highlighted in Fig. 2. In quantitative analysis of organic compounds, the desirable signal is usually a singlet that is not subject to exchange phenomena and is well resolved from any other signal arising from the sample. In addition, intensity of the selected peak should be larger than most other signals of the compound in order to improve the detection limit and sensitivity (Shamsipur *et al.*, 2002). Therefore, we selected two signals for artemisinin at δ 5.86 and 1.444, and three singlet signals for camphor at δ 0.961, 0.910 and 0.835. This selection was made taking into consideration that all of the signals were in a non-crowded region and were well separated from each other.

The next step was the evaluation of different compounds as possible internal standard, since quantitative NMR requires addition of an internal standard in order to calculate the amount of analyte present in the sample by comparison of the integral ratios (Pauli *et al.*, 2005). The ^{1}H-NMR signal of *tert*-butanol does not overlap with any resonances of the extract, shows a sharp singlet at δ 1.276 (9-H), is soluble and stable in sample media and hence represents a good chose for this analysis.

The determination of relaxation delay (d_1) value is the most important factor since insufficient relaxation results in the underestimation of the amount of compound present in the samples. For a 90° pulse, the d_1 delay should be five times T_1(max), where T_1(max) relates to the proton with the longest relaxation time in the sample, but not necessarily from the target analyte (Pauli *et al.*, 2005).

In order to use ^{1}H-NMR as a method for artemisinin and camphor quantification, calibration curves were obtained for both artemisinin and camphor. For each pure compound, five diluted solutions were prepared from the stock solution. For artemisinin, the range of concentrations used was 9.85–97.77 mM and for camphor it was 10.09–100.85 mM. Expressing the ratio I_C/I_T, where I_C is the integral value of the protons of each compound and I_T is the integral value of the protons of the internal standard, as a function of the concentration of each compound allowed the creation of calibration plots for artemisinin and camphor (Fig. 3). The linearity of response was determined from the linear determination factors R^2, which were 0.9968 (δ 5.864) and 0.9986 (δ 1.444) for the artemisinin peaks, and 0.999 (δ 0.961), 0.998 (δ 0.910) and 0.999 (δ 0.835) for

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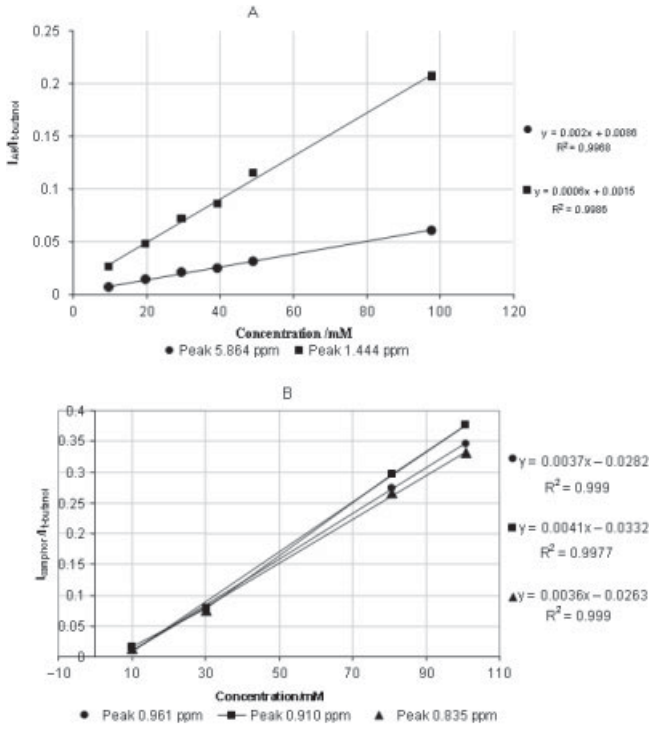

Figure 3 Calibration curves for artemisinin and camphor showing (A) $I_{artemisinin}/I_{butanol}$ vs. concentration of artemisinin, and (B) $I_{camphor}/I_{butanol}$ vs. concentration of camphor.

the camphor peaks. All the peaks mentioned could be used in the quantification method, but those at δ 5.864 for artemisinin and at δ 0.910 for camphor were chosen since these signals suffered from less interference (i.e. not or only slightly overlapped) by those of other components, as shown in Fig. 2. The ^{1}H-NMR spectra of the standard mixture of artemisinin and camphor was obtained and the concentrations of each component derived from the calibration curves. Results were coincident within a range of 3.36 and 1.53% for artemisinin and camphor, respectively. These results indicate that quantification of artemisinin and camphor by ^{1}H-NMR can be carried out with the use of spectra acquired using the selected parameters.

^{1}H-NMR spectra of acetone extracts of *A. annua* were obtained under the same experimental conditions as described above, and the concentration of the two major components estimated from the calibration curves. We determined the optimal amount of extract (100 mg) to be diluted in 1 mL of deuterochloroform containing 6.52 mg of *tert*-butanol in order to observe good solubility and reproducible chemical shift values in the spectra of pure compounds and of the mixtures. The analyses of five *A. annua* acetone extracts (three replicate samples for each extract) independently obtained are reported in Table 1. A further validation of our new method ^{1}H-NMR quantification was achieved by the comparative analysis of samples 1 and 2 (Table 1) using an HPLC-RI technique (Redher *et al.*, 2002). The results of 0.72 and 0.71%, respectively, were fairly similar to those obtained by ^{1}H-NMR. From all of these studies we can assert that our method of quantification is well validated and gives reproducible results.

In preliminary studies, we compared petroleum ether 40–60, *n*-hexane, cyclohexane, dichloromethane, chloroform, methanol and ethanol as solvents for the extraction of artemisinin. The highest yields of extraction of artemisinin were obtained with chloroform. It was noticeable that the extraction rate and the yields of artemisinin increased with the increment of the dielectric constant of the solvent. However, chloroform and both alcohols, although effective at extracting artemisinin, also extracted a lot of other more polar components (unpublished results) and thus showed a very poor selectivity towards the active principle.

In the present work, we found that acetone has an extracting capacity towards artemisinin similar to that of chloroform, but exhibits a much higher selectivity, as shown by TLC. Soxhlet extraction can be an alternative extracting method that makes use of lower amounts of solvent, but it has no significant impact on the yield of the extraction, as we and previous workers (Hao *et al.*, 2002) have found, and requires heat and

Table 1. Quantification of artemisinin and camphor in different extracts of leaves of *Artemisia annua* hybrid CPQBA × POP by ^{1}H-NMR

	Artemisinin		Camphor	
Sample	Percentage in extract[a]	Percentage in plant[b]	Percentage in extract[a]	Percentage in plant[b]
1	27.684 ± 1.307	0.77	4.958 ± 0.070	0.14
2	29.471 ± 1.602	0.86	4.753 ± 0.255	0.13
3	28.730 ± 0.953	1.06	5.138 ± 0.121	0.19
4	23.396 ± 0.215	1.06	4.574 ± 0.368	0.19
5	25.314 ± 0.326	0.98	4.757 ± 0.245	0.18

[a] Calculated percentages shown are mean value ± standard error (n = 3).
[b] Determined using the mean values of concentration in extract.

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special (expensive and size-limited) apparatus. Acetone has additional advantages of low toxicity, low cost, low boiling point and low environmental impact, which make it a suitable solvent for the extraction *in situ*, i.e. in countries where anti-malarial substances are required.

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REFERENCES

Abu-Raddad LJ, Patnaik P, Kublin JG. 2006. Dual infection with HIV and malaria fuels the spread of both diseases in sub-Saharan Africa. *Science* **314**: 1603–1606.

Blasko G, Cordel GA, Lankin DC. 1988. Definitive ′H- and ^{13}C-NMR assignments of Artemisinin (Qinghaosu) *J Nat Prod* **51**: 1273–1276.

Brown GD. 2003. ^{13}C-^{2}H Correlation NMR spectroscopy studies of the *in vivo* transformations of natural products from *Artemisia annua*. *Phytochem Rev* **2**: 45–59.

Charles DJ, Simon JE, Wood KV, Heinstein P. 1990. Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extracts. *J Nat Prod* **53**: 157–160.

Chen HL, Wang KT, Pu QS, Chen XG, Hu ZD. 2002. On-line conversion and determination of artemisinin using a flow-injection capillary electrophoresis system. *Electrophoresis* **23**: 2865–2871.

Christen P, Veuthey JL. 2001. New trends in extraction, identification and quantification of artemisinin and its derivatives. *Curr Med Chem* **8**: 1827–1839.

Dhingra V, Vishweshwar Rao K, Lakshmi Narasu M. 2000. Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sci* **66**: 279–300.

El-Sohly HN, Croom EM, Jr, El-Feraly FS, El-Sherei MM. 1990. A large-scale extraction technique of artemisinin from *Artemisia annua*. *J Nat Prod* **53**: 1560–1564.

Ferreira JF, Janick J. 1996. Immunoquantitative analysis of artemisinin from *Artemisia annua* using polyclonal antibodies. *Phytochemistry*. **41**: 97–104.

Guerin PJ, Olliaro P, Nosten F, Druilhe P, Laxminarayan R, Binka F, Kilama WL, Ford N, White NJ. 2002. Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. *Lancet Infect Dis* **2**: 564–573.

Hao J-Y, Han W, Huang S-D, Xue B-Y, Deng X. 2002. Microwave-assisted extraction of artemisinin from *Artemisia annua* L. *Sep Purif Technol* **28**: 191–196.

Jaziri M, Diallo B, Vanhaelen M, Homès J, Yoshimatsu K, Shimomura K. 1993. Immunodetection of artemisinin in *Artemisia annua* cultivated in hydroponic conditions. *Phytochemistry* **33**: 821–826.

Klayman DL. 1985. Qinghaosu (artemisinin): an antimalarial drug from China. *Science* **228**: 1049–1055.

Kohler M. 1999. Extraction et Analyse des Principes Actifs D'origine Végetale. Application à L'artémisinine Extraite de Artemisia annua L. PhD thesis no. 3129. University of Geneva.

Kohler M, Haerdi KW, Christen P, Veuthey JL. 1997. Extraction of artemisinin and artemisinic acid from *Artemisia annua* L. using supercritical carbon dioxide. *J Chromatogr A*, **785**: 353–360.

Lapkin AA, Plucinski PK, Cutler M. 2006. Comparative assessment of technologies for extraction of artemisinin. *J Nat Prod* **69**: 1653–1664.

Magalhães PM, Pereira B, Sartoratto A. 2004. Yields of antimalarial *Artemisia annua* L. species. *Acta Hort* **629**: 421–424.

Mutabingwa TK, 2005. Artemisinin-based combination therapies (ACTs): best hope for malaria treatment but inaccessible to the needy! *Acta Trop.* **95**: 305–315.

Olliaro PI, Taylor WR. 2004. Developing artemisinin based drug combinations for the treatment of drug resistant falciparum malaria: a review. *J Postgrad Med* **50**: 40–44.

Pauli GF, Jaki BU, Lankin DC. 2005. Quantitative ^{1}H NMR: development and potential of a method for natural products analysis. *J Nat Prod* **68**: 133–149.

Price R, Van Vugt M, Phaipun L, Luxemburger C, Simpson J, McGready R, Kuile FT, Kham A, Chongsuphajaisiddhi T, White NJ, Nosten FO. 1999. Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives. *Am J Trop Med Hyg* **60**: 547–555.

Rehder VLG, Rodrigues MVN, Foglio MA, Sartoratto A. 2002. Dosagem de artemisinina em *Artemisia annua* L. por cromatografia líquida de alta eficiência com detecção por indice de refração. *Rev Bras Farmacog* **12S**: 116–118.

Robert A, Dechy-Cabaret, Cazelles J, Bernard Meunier. 2002. From mechanistic studies on artemisinin derivatives to new modular antimalarial drugs. *Acc Chem Res* **35**: 167–174.

Schmid G, Hofheinz W. 1983. Total synthesis of Qinghaosu. *J Am Chem Soc* **105**: 624–625

Shamsipur M, Talebpour Z, Bijanzadeh HR, Tabatbaei S. 2002. Monitoring of ampicillin and its related substances by NMR. *J Pharm Biomed Anal* **30**: 1075–1085.

Van Geldre E, Vergauwe A, Van den Eeckaut E. 1997. State of the art of the production of the antimalarial compound artemisinin in plants. *Plant Mol Biol* **33**: 199–209.

Van Nieuwerburgh FC, Vande Casteele SR, Maes L, Goossens A, Inze D, Van Bocxlaer J, Deforce DL. 2006. Quantitation of artemisinin and its biosynthetic precursors in *Artemisia Annua* L. by high performance liquid chromatography–electrospray quadrupole time-of-flight tandem mass spectrometry. *J Chromatogr A* **1118**: 180–187.

Wallaart TE, van Uden W, van Lubberink HGM, Woerdenbag HJ, Pras N, Quax WJ. 1999. Isolation and identification of dihydroartemisinic acid from *Artemisia annua* and its possible role in the biosynthesis of artemisinin. *J Nat Prod* **62**: 430–433.

Wang M, Park C, Wu Q, Simon JE. 2005. Analysis of artemisinin in *Artemisia annua* L. by LC-MS with selected ion monitoring. *J Agric Food Chem* **53**: 7010–7013.

WHO. 2005. Roll back malaria. World malaria report: section III global financing, commodities and services delivery. World Health Organization: Geneva. Available at: http://www.rbm.who.int/wmr2005/htm

WHO. 2006. WHO monograph on good agricultural and collection practices (GACP) for *Artemisia annua* L. World Health Organization, Geneva.