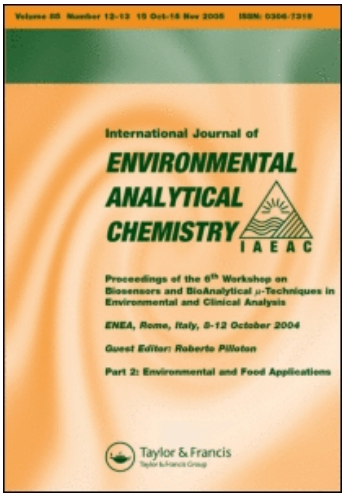


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Karina K. Jessing[a]; Thomas Bowers[ab]; Bjarne W. Strobel[a]; Bo Svensmark[b]; Hans Christian Bruun Hansen[a]
[a] Faculty of Life Sciences (LIFE), Department of Basic Sciences and Environment, University of Copenhagen, Frederiksberg, Denmark [b] Faculty of Science (NAT), Department of Chemistry, University of Copenhagen, Copenhagen, Denmark

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# Artemisinin determination and degradation in soil using supercritical fluid extraction and HPLC-UV

Karina K. Jessing[a], Thomas Bowers[ab], Bjarne W. Strobel[a]*, Bo Svensmark[b] and Hans Christian Bruun Hansen[a]

[a]*Faculty of Life Sciences (LIFE), Department of Basic Sciences and Environment, University of Copenhagen, Frederiksberg, Denmark;* [b]*Faculty of Science (NAT), Department of Chemistry, University of Copenhagen, Copenhagen, Denmark*

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Artemisinin, a bioactive compound produced in *Artemisia annua* L. (sweet wormwood) is used as active ingredient in drugs against malaria. Cultivation of *A. annua* at field scale implies high amounts of artemisinin produced and potential high losses to soil with impact to vulnerable organisms in soil and leaching to the aquatic environment. A new method was developed for extraction of artemisinin in sandy, clayey and humic soil samples by supercritical fluid extraction (SFE) and determination by HPLC. Optimal SFE conditions were reached using ethanol as modifier at a flow of $0.5\,mL\,min^{-1}$ and a total extraction time of 20 min. The HPLC method had linearity up to $>535\,mg\,kg^{-1}$ for all soil types, limit of detection (LOD) was $13\,\mu g\,kg^{-1}$ soil and limit of quantification (LOQ) was $43\,\mu g\,kg^{-1}$ soil. Recovery for soil samples spiked with artemisinin 1 h before extraction was determined to 70–80%. No matrix effect was observed in the detection. The method enabled quantification of artemisinin in three common soil types, and was applied for determination of degradation kinetics of artemisinin in spiked soils. Degradation kinetics consisted of an initial fast degradation followed by a slower one. The slower reaction could be fitted by first-order kinetics resulting in rate constants of 0.05, 0.084 and 0.32 per day in sandy, clayey and humic soil, respectively. Both the rate of the fast and slow reaction appeared to increase with soil organic matter content. The relative long persistence time in soil increases the risk of toxic effects on non target organisms in soil as well as in water.

**Keywords:** artemisinin; biomedicine; malaria; natural toxins; qinghaosu; SFE; sesquiterpene

## 1. Introduction

Artemisinin, a sesquiterpene lactone with an endoperoxide bridge [1] (Table 1), has had great attention the last three decades due to antimalarial [2] and possible antitumour properties [3]. Artemisinin is synthesised and accumulated in the plant *Artemisia annua* L. (Sweet wormwood) [4]. Artemisinin is now available commercially as an antimalarial drug efficacious against drug-resistant strains of *Plasmodium*, the malaria parasite. Since chemical synthesis or *in vitro* production of artemisinin at present is not commercially

*Corresponding author. Email: bjwe@life.ku.dk

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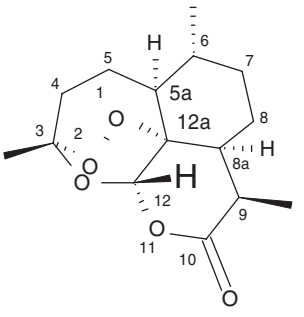
Table 1. Chemical structure and selected properties of artemisinin.

| | |
|---|---|
| CAS no. | 63968-64-9 |
| Synonyms | Artemisinine, qinghaosu |
| IUPAC name | (3*R*, 5a*S*, 6*R*, 8a*S*, 9*R*, 12*S*, 12a*R*)-octahydro-3, 6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4, 3-j]-1, 2-benzodioxepin-10(3*H*)-one |
| Chemical structure |  |
| Molecular formula | $C_{15}H_{22}O_5$ |
| Molar mass | $282.2\,g\,mol^{-1}$ |
| Solubility in water | $49.7 \pm 3.7\,mg\,L^{-1}$ [b] |
| Log $K_{OW}$ | 2.90[a] |
| Log $K_{OC}$ | 2.51[a] |
| Henrys law constant | $4.92 \times 10^{-9}\,atm\,m^3\,mol^{-1}$ [a] |

Notes: [a]Calculated with EPIwin v3.12 (US EPA). [b]Jessing *et al*. 2008 [14].



feasible [5], *A. annua* is cultivated in large scale for production of malaria medicine as 40% of the world population is threatened by malaria [6].

The bioactive properties of artemisinin are due to the endoperoxide bridge. Artemisinin is one of the very few naturally occurring endoperoxides. The mechanism of action in *Plasmodium* parasites comprise reductive cleavage of the peroxide bond, facilitated by $Fe^{II}$, leading to formation of oxygen-centred radicals, which in turn, can transform to carbon-centred radicals [7]. This activated intermediate can form covalent adducts with specific parasite membrane-associated proteins [8]. It is also most likely that artemisinin react with amino acids, proteins, amino sugars, enzymes and dissolved humic substances present in soil (in the following referred to as SOM). In addition, artemisinin has insecticidal [9,10] and phytotoxic properties [11–13]. Cultivation of *A. annua* L. may contaminate the soil with artemisinin, and recent studies revealed that artemisinin at realistic soil concentrations of $5.24\,mg\,kg^{-1}$ repelled earthworms, causing decrease of soil quality [14]. Jessing *et al*. [14] found that the growth of salad, used as a representative of a crop following *A. annua*, was inhibited by 50% at a soil concentration of $2.48\,mg\,kg^{-1}$.

Proper description of the fate, exposure and toxicity of artemisinin require a simple, exact and fast method for artemisinin extraction from soil. Supercritical $CO_2$ has solvent properties similar to hexane, a relatively apolar solvent [15] and the relatively apolar compound artemisinin is likely to be extracted effectively using supercritical fluid extraction (SFE) with $CO_2$. Numerous applications of SFE to various natural product classes have been published, e.g. carotenoids [16], terpenoids from *Mentha piperita* [17] and alkaloids such as caffeine [18]. SFE is widely used in extraction of pesticides with similar chemical properties as artemisinin, e.g. atrazine [19], fluometuron [20] and sulfonylurea herbicides

[21] in soil. In addition, SFE methods for extracting artemisinin in plant tissue have been developed. Artemisinin from aerial parts of *A. annua* can be quantitatively extracted in 20 min using a supercritical fluid mixture of carbon dioxide and 3% (v/v) ethanol [22,23]. A large advantage of SFE compared to liquid extraction, which is the most common way of extracting artemisinin out of plant material, is the minimised use of organic solvents [23].

Artemisinin can be released to soil either via dead plant material, leaching from leaves by rain or incorporation of plant parts left over after harvest. A simple extraction method of artemisinin from soil is required to investigate the persistence of this bioactive compound in soil. Determination of the persistence of artemisinin in soil is important regarding toxicity and risk of leaching. Generally natural toxins are easily degraded in soil. The sesquiterpene lactone toxin parthenin produced by *Parthenium hysterophorus* L. had a half life of 59 h in topsoil at 20°C and biotic degradation was strongly indicated [24]. Parthenin is a sesquiterpene lactone without a peroxide bridge. The hydrolysis of other natural toxins such as cyanogenic glucosides and glucosinolates and the following degradation of their toxic metabolites are within the range of a few days [25,26].

In this study, an extraction method using SFE is developed for three common soil types with different clay and humic matter contents. The developed extraction method was applied to artemisinin degradation kinetics with the three soils.



## 2. Experimental

### 2.1 *Chemicals*

Artemisinin of 98% purity was provided by Sigma Aldrich. Ethanol (96%) was provided by Kemetyl, Køge, Denmark. Methanol and acetonitrile, both of HPLC grade were provided by Sigma Aldrich. Sodium hydroxide (NaOH) was provided by J.T. Baker, acetic acid (>99.8% pure), $Na_2HPO_4$ and $NaH_2PO_4$ were provided by Merck. Compressed $CO_2$ (99.998% purity) was provided by Hede Nielsen, Taastrup, Denmark.

### 2.2 *Soils*

Three representative agricultural topsoils from Denmark were sampled. The soils comprised a sandy soil from Jyndevad developed on glaciofluvial material and classified as a Humic Psammentic Dystrudept [27], a clayey soil from Sjællands Odde developed on calcareous clayey lodgement and melt-out till from the Weichsel glaciation and classified as a Typic Agriudoll [27], and a humic-rich soil from Tybjerg near Ringsted developed in a depression in the sandy out-wash plains and classified Histosol. Selected characteristics of the soils used are shown in Table 2. In the following, the soils are referred to as sandy, clayey and humic soils. Soil material was sampled from the A horizons, air-dried and passed though a two mm sieve. All soils have about neutral pH. The content of *C* in humic matter differs and is substantially higher in the humic soil. In addition, the humic soil has the highest content of CBD (Citrate–Bicarbonate–Dithionite) and oxalate extractable Fe and Al. The lowest ratio between oxalate and CBD extractable Fe observed for the sandy soil reflects that the Fe oxides are more crystalline in this soil compared with the other soils.

The soils used in the method development were spiked dry with a known amount of artemisinin dissolved in ethanol. The spiked soil is left for approximately 2 h allowing the artemisinin to incorporate in soil before extraction.

Table 2. Selected characteristics for the three soils.

| | | % | | | | | | | ($mg\,kg^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Soil type | Depth (cm) | C[a] | N[b] | Clay[c] | Silt[c] | Sand[c] | pH[d] | WHC[e] | $Fe^{f}_{ox}$ | $Al^{f}_{ox}$ | $Fe^{g}_{CBD}$ | $Al^{g}_{CBD}$ |
| Sandy | 10–25 | 2.4 | 0.12 | 5 | 3 | 92 | 6.9 | 30 | 1440 | 1010 | 2220 | 1030 |
| Clayey | 0–30 | 2.2 | 0.23 | 19 | 18 | 63 | 7.2 | 38 | 1760 | 750 | 4080 | 760 |
| Humic | 0–30 | 6.7 | 0.2 | 9 | 22 | 69 | 6.3 | 63 | 3600 | 2000 | 6300 | 1250 |

Notes: [a]Was determined by dry combustion.
[b]Was determined by the Kjeldahl method.
[c]Clay $<2\,\mu m$, silt 2–20 μm and sand $>20\,\mu m$ determined by the hydrometer method and sieving
[d]Measured in 0.01 M $CaCl_2$.
[e]After complete water saturation and free draining for 2 h, WHC (water holding capacity) determined by (dry weight wet weight/dry weight) $*$ 100. Dry weight was determined by drying the soil in oven at 105°C for 24 h.
[f]Oxalate-extractable Fe and A1.
[g]Citrate–Bicarbonate–Dithionite extractable Fe and Al.



### 2.3 *Supercritical fluid extraction*

In the final method, 10 g dry soil material was packed in a 24 mL extraction cell (14 × 150 mm, Applied Separations) with glass fibre filter (Advantec) in both bottom and top. Artemisinin was then extracted by dynamic SFE on a Spe-ed SFE (Applied Separations, Allentown, PA, USA) at 500 bars and 50°C using $CO_2$ as fluid with a flow of $1.5\,mL\,min^{-1}$ as described by Kohler *et al.* [23]. Ethanol was used as a modifier with a flow of $0.5\,mL\,min^{-1}$ (3% v/v) and the extraction time was 20 min. The elute was collected in ethanol (96%) and evaporated to dryness under a stream of cleansed compressed air.

#### 2.3.1 *SFE method optimising*

Kohler *et al.* [22] stated methanol or ethanol as the best modifier when extracting artemisinin from *A. annua* plant material. Ethanol was chosen as modifier in our experiments as it is less toxic than methanol. A modifier flow of 0, 0.5 and $1.0\,mL\,min^{-1}$ was tested. The influence of extraction time was tested in an experiment with 20, 40 and 60 min of extraction. The different test conditions were evaluated in steps combined in sequences as described in Figure 1.

It was tested whether interfering compounds from the soil were extracted with artemisinin in an experiment comparing yield of artemisinin from spiked soil and spiked glass fibre.

### 2.4 *Quantification of artemisinin*

Determination of artemisinin was performed using the procedure developed by Zhao and Zeng [28] and optimised by Qian *et al.* [29] where artemisinin was converted to the strongly UV-absorbing compound Q260 by a pre-column reaction. The procedure described briefly: dry extract from SFE was dissolved in 1 mL 96% ethanol and treated with 4 mL 0.2% NaOH (w/v) at 50°C for 30 min and cooled to room temperature. The solution was then acidified with 5 mL 0.08 M acetic acid and filtered through a Millipore filter (0.45 μm)

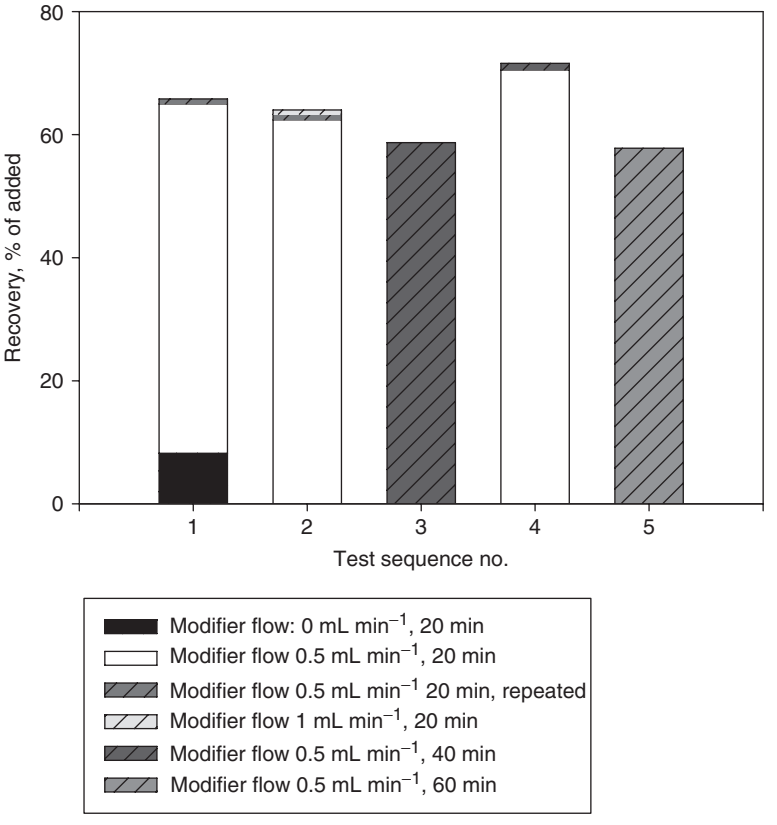



Figure 1. Accumulated recovery as % of added artemisinin in five sequences of different 3-step combinations of ethanol modifier flow and time tested.



before determination by HPLC using an Agilent 1100 series. The samples were separated in a Supelco Discovery Bio C18 Bio Wide Pore column (25 cm × 4.6 mm, 5 μm) fitted with a Supelco Discovery Bio Wide Pore C18 guard column (2 cm × 4.0 mm, 5 μm) (Sigma-Aldrich). The column temperature was 30°C. The mobile phase was methanol/acetonitrile/0.9 mM $Na_2HPO_4$–3.6 mM $NaH_2PO_4$ buffer (pH 7.76) solution (45/10/45 v/v) and the injection volume was 20 μL. The elution speed was 1 mL $min^{-1}$ and the detection wavelength 260 nm. Quantification was unaffected by soil matrix as there were no interfering peaks or reactants (Figure 2).

### 2.5 *Degradation of artemisinin in soil*

In the degradation kinetic experiments, the soils were rewetted to 40% of WHC (water holding capacity) and incubated for 1 week before adding the remaining water up to 60% of WHC. Then the soils were spiked in the following way; 10 mL stock solution (0.64 mg $mL^{-1}$) of artemisinin dissolved in ethanol was added to 10–20 g soil and mixed well, then another 20–40 g soil was mixed in and so on up to 640 g moistened soil to ensure homogeneous mixing. The spiked soil was distributed equally to two cylindrical glass

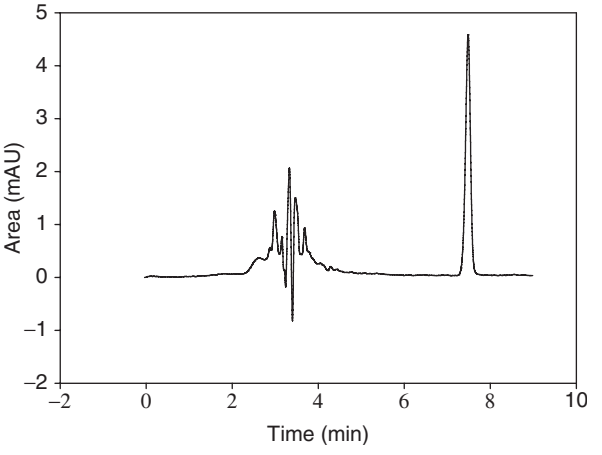



Figure 2. Chromatogram of artemisinin extracted from clayey soil using the final SFE method. Artemisinin has retention time of 7.5 min.



beakers (2 L) run as replicates. The initial artemisinin concentration was 16.67 mg $kg^{-1}$ DW (dry weight basis). The beakers were sealed with wrapping film and placed in the dark at 22°C. Oxygenation was allowed at each sampling time. Four replicates from each soil were sampled 1 h after spiking and at day 1, 2, 3, 4, 7, 9, 11, 14, 18, 23, 28, 35 and 51. The beakers were weighted during the experiment and water was added to compensate for water loss.

## 3. Results and discussion

### 3.1 *Development and optimising of the SFE method*

Ethanol was used as modifier and the temperature was set to 50°C based on the results obtained by Kohler *et al.* [23]. The pressure was set to 500 bar and the $CO_2$ flow at 1.5 mL $min^{-1}$, while the modifier flow was varied. The use of modifier has a pronounced effect as seen from procedure one where exclusion of the modifier extracted only 8% (Figure 1). Using a modifier flow of 0.5 mL $min^{-1}$ increased the recovery to 67%. Further increase of the modifier flow to 1 mL $min^{-1}$ in sequence no. 2 did not increase recovery, and prolonged extraction time gave no improvements in sequence no. 3, 4 and 5. Extraction time of 20 min with a modifier flow of 0.5 mL $min^{-1}$ is sufficient as seen for step one in sequence 2 (Figure 1).

### 3.2 *Validation*

The recovery of the optimised method was 74–84% (Table 3) depending on soil type. The best recovery was obtained in the sandy soil which can be explained by low contents of SOM (soil organic matter). It is likely that artemisinin reacts quickly with organic substances in the soil which may explain the relatively low recovery for the humic soil. Jessing *et al.* [14] reported similar observations. This is supported by the data from the experiment where soil was substituted with glass fibre as matrix resulting in a recovery close to 100% (Table 3). It is known that pesticides form a certain proportion of

Table 3. Recoveries ± relative standard deviation of artemisinin extraction in three different soils and inert glass material, using the final SFE method (20 min, ethanol modifier flow: 0.5 mL $min^{-1}$) on air dry soil.

| Soil | *n* | Recovery ± RSD (%) |
|---|---|---|
| Clayey | 6 | 79 ± 3.0 |
| Sandy | 7 | 84 ± 2.0 |
| Humic | 6 | 74 ± 4.7 |
| Glass fibre | 3 | 96 ± 3.6 |

Note: Added start concentration 23.4 mg $kg^{-1}$.

non-extractable residues in soil [30]. This fraction of non-extractable residues depend among other things on soil content of organic matter [31] and this might explain why full recovery was not obtained in any of the soils.

Recoveries were almost the same in a concentration range from 6.2 to 27.4 mg $kg^{-1}$. Limit of linearity was tested up to 535 mg $kg^{-1}$ soil and the method is valid in a broad range of concentration.

Limit of detection (LOD) in soil was determined as three times standard deviation ($s = 0.00176$) of seven measurements on a sample containing 0.41 μg $mL^{-1}$ and the LOQ was determined as 10 times standard deviation. The LOD was 0.013 μg $mL^{-1}$ (12.8 μg $kg^{-1}$ soil) while the LOQ was determined to 42.5 μg $kg^{-1}$ soil. In these calculations, the concentration of the measured samples is taken into account.



### 3.3 *Stability of Q260*

The stability of the strongly UV-absorbing compound Q260 in aqueous solution was evaluated over a period of nine days. All spectrophotometric measurements revealed the same spectra and absorbance and converted artemisinin was concluded stable for at least 9 days.

### 3.4 *Degradation kinetic experiment*

The developed extraction method was applied to degradation kinetic experiments in the three different soils (Figure 3).

Degradation was modelled using standard first-order decay expression with two parameters (Equation (1)):

$$[\text{art}] = [\text{art}]_0 \, e^{-bt} \tag{1}$$

Where [art] is the residual artemisinin in the soil in percentage of the initial added amount, $[\text{art}]_0$ is the estimated artemisinin content at time zero in percentage of the initially added amount, $b$ is the rate constant and $t$ is the time in days (see Table 4 for model estimates).

In all three soils, degradation kinetics consisted of a fast initial process followed by a slower one. Degradation kinetics was modelled on the second slower process. Degradation of artemisinin in soil strongly depends on soil characteristics. Comparison of the rate constants in the three soils ($b$ in Table 4) reveals that degradation in the humic soil is 3.8 times faster than in the clayey soil, which in turn is 1.7 times faster than degradation

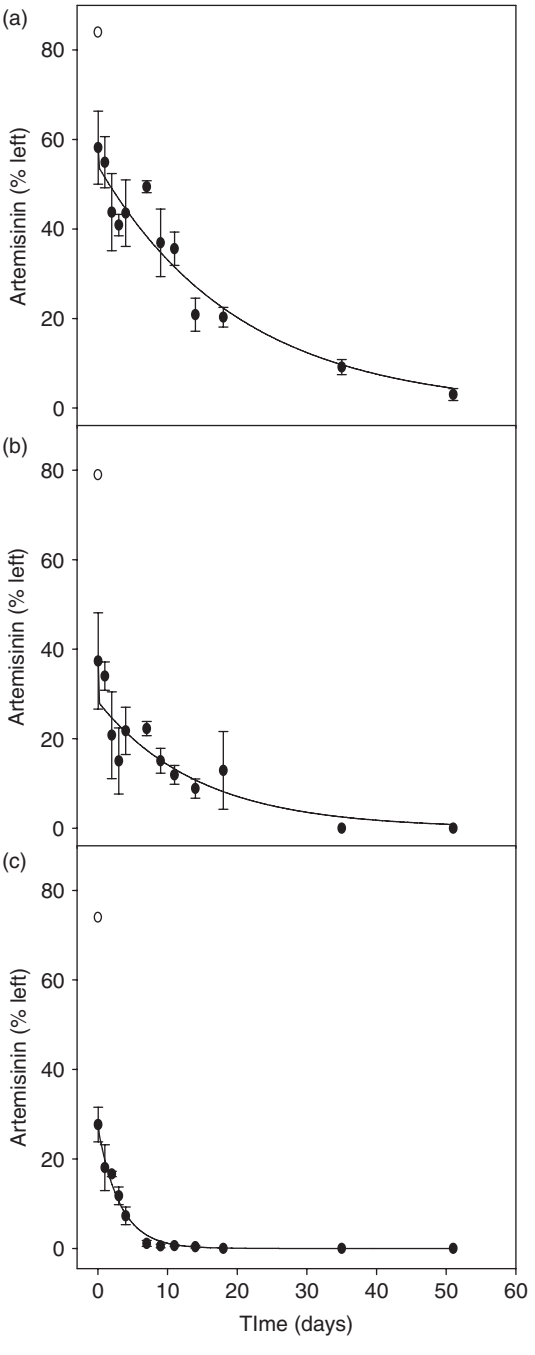



Figure 3. Degradation in (a) sandy soil, (b) clayey soil and (c) humic soil at 60% of WHC and 22°C. Artemisinin in percent left of initial added concentration as a function of time in days, given as average of four replicates (error bars represents standard deviations). The open circles represent recovery at time zero. Full line is due to fitting with Equation (1) (see fitting parameters in Table 4).

Table 4. Degradation kinetic parameters and half lives of artemisinin in sandy, clayey and humic soils, at 22°C and 60% of WHC.

| Soil | $[art]_0$ (%) | $b$ ($d^{-1}$) | $r^2$ | $t_{1/2}$ ($d^{-1}$) |
|---|---|---|---|---|
| Sandy | $55.4 \pm 1.86$ | $0.05 \pm 0.005$ | 0.92 | 13.5 |
| Clayey | $31.8 \pm 2.14$ | $0.084 \pm 0.014$ | 0.83 | 8.28 |
| Humic | $27.8 \pm 0.95$ | $0.32 \pm 0.022$ | 0.97 | 2.14 |

in the sandy soil. The degradation rate in the humic soil is 6.4 times faster than the one in the sandy soil (Figure 3). In addition, the fraction of artemisinin disappeared in the initial process is larger in the humic than the sandy soil.

Two factors may explain the fast degradation in humic soil. First, this soil had the highest content of organic matter (Table 2) to which artemisinin can sorb strongly or possibly react with. Second, this soil has a high content of iron oxides. In biological systems, artemisinin reacts with $Fe^{2+}$ [7] and degradation enhanced by mineral surfaces may play a role. $Fe^{2+}$ can act as a catalyst in cleavage of the peroxide bridge [7]. However, as all soils are aerobic, no $Fe^{2+}$ is present in solution and other catalysts may be present such as $Fe^{3+}$ and $Mn^{2+}$ at soil mineral surfaces. It is remarkable that a large fraction of the added artemisinin already has disappeared shortly after addition at the time zero. Probably, a large fraction of the added artemisinin has reacted irreversibly with SOM. This fast initial process is then followed by a slower degradation process, the one we actually see in Figure 3. Degradation half lives in soil for the slower process were 13.5, 8.3 and 2.1 days in sandy, clayey and humic soil, respectively. Again it is seen that the fastest degradation is obtained for the soil having the highest SOM content, indicating that SOM reacts irreversible with artemisinin and/or that SOM stimulates microbial activity causing faster degradation. The half lives of artemisinin in soil are longer than the ones of other natural toxins, e.g. parthenin (59 h) [24] and cyanogenic glucosides and glucosinolates (few days) [25,26]. The endoperoxidic compound seems to be quite stable in soil compared to these other natural toxins. This relative long persistence time in soil increase the risk of toxic effects on non-target organisms in soil as well as in water. The estimated $K_{oc}$ of $324\ L\ kg^{-1}$ (Table 1) indicates medium mobility of artemisinin in soil and hence, the risk of leaching is present.



## 4. Conclusion

A SFE method has been developed with acceptable recoveries in three common soils with very different soil characteristics. Recoveries were: in clayey soil 79%, in sandy 84% and in humic soil 74%. LOD of the method is $12.8\ \mu g\ kg^{-1}$ soil. The SFE method provided extracts without any interfering substances, a large advantage when extracting from soil material.

The developed SFE method was successfully applied on degradation kinetic experiments. Apparently disappearance of artemisinin in soil depends on soil characteristics. High content of substances like humic matter and clay results in faster initial disappearance. The processes behind the slower degradation hereafter can be both biotic and abiotic.

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