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Monitoring of Artemisinin, Dihydroartemisinin, and Artemether in Environmental Matrices Using High-Performance Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS)

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ABSTRACT: The area cultivated with *Artemisia annua* for the extraction of the antimalarial compound artemisinin is increasing, but the environmental impact of this cultivation has not yet been studied. A sensitive and robust method using liquid chromatography−tandem mass spectrometry (LC-MS/MS) was developed for the determination of artemisinin in soil. Dihydroartemisinin and artemether were included in the method, and performance on analytical columns of both traditional C_{18} phenyl-hexyl and porous shell particles-based Kinetex types was characterized. The versatility of the method was demonstrated on surface water and groundwater samples and plant extracts. The limit of detection was 55, 30 (25 ng/g soil), and 4 ng/mL for dihydroartemisinin, artemisinin, and artemether, respectively. Method performance was demonstrated using naturally contaminated soil samples from *A. annua* fields in Kenya. The highest observed concentrations were above EC_{10} for lettuce growth. Monitoring of artemisinin in soil with *A. annua* crop production seems necessary to further understand the impact in the environment.

KEYWORDS: *Artemisia annua*, African soils, environment, antimalarial, natural contaminants, biomedicine, qinghaosu, *Plasmodium falciparum*

INTRODUCTION

The medicinal plant *Artemisia annua*, also known as Sweet Wormwood, Sweet Annie, Sweet Sagewort, or Annual Wormwood (Chinese,青蒿; pinyin, qīnghāo), is cultivated at large scale in Asia and the Middle East for medicinal purposes, and in Africa, cultivation is in the establishing phase.[1] *A. annua* produces artemisinin (Figure 1, **1**), a sesquiterpene lactone with a endoperoxide bridge and an efficient drug against chloroquine-resistant strains of *Plasmodium falciparum* in the treatment of malaria.[2] Since 2005, **1** combination therapies (ACTs) have been the recommended treatment for malaria by the WHO and are extensively used worldwide.[3] At present, chemical synthesis[4] or in vitro production of **1** is not feasible economically,[5] and cultivation of the plant is still the only valid source of **1**.[6] Thus, *A. annua* has to be cultivated at a large scale to satisfy the vast need for medicine, as 40% of the world's population is threatened by malaria.[7] In addition to antimalarial activity, **1** and semisynthetic derivatives have activity against cancer cells, schistosomiasis, and various viral diseases.[8] Therefore, there is a need for a more widespread cultivation of *A. annua* as a medicinal source in additional regions to satisfy the high demands.

During the cultivation of *A. annua*, some of the **1** produced is released to the soil environment, where it may have negative effects on the soil environment, including groundwater resources. As **1** has a water solubility of 50 mg/L and estimated log K_{OW} of 2.9, medium mobility in soil is anticipated, and there is a risk of leaching to groundwater resources.[9] Previous studies on fields used for the production of *A. annua* indicated a possible risk of lower yield of the following crop and negative effects on soil fauna. Lettuce growth (EC_{10} 0.54 ± 0.32 mg/kg) as well as the number of earthworms decreased with increasing levels of **1** in the soil (EC_{10} 5.24 ± 2.64 mg/kg).[9] Duke et al.[10] found that **1** inhibited the germination of lettuce seeds at a concentration of 0.93 μg/L in a Petri dish experiment. Surface runoff water contaminated with **1** may also pose an environmental risk, as **1** has been shown to affect freshwater algae, such as *Pseudokirchneriella subcapitata* and the macrophyte *Lemna minor*, adversely at low concentrations (EC_{50} = 0.24 and 0.19 mg/L, respectively).[9] Summing up the present state of knowledge on possible risks associated with the release of **1** during the cultivation of *A. annua*, it seems clear that further studies are required. For this purpose, techniques for monitoring **1** in environmental matrices are needed, to reveal the fate of the compound and establish knowledge on the concentrations of **1** under natural growing conditions. Such information is of foremost importance for risk evaluation and assessment of possible environmental side effects caused by the cultivation of *A. annua*. A number of methods have been published for the analysis of **1** in drugs and biological matrices.[11−13] Because of the complexity of soil extracts, such methods are not straightforwardly transferred to environmental fate studies, and dedicated methods needs to be developed. So far, quantitation of **1** in soil extracts has been made using high-performance liquid chromatography with ultraviolet detection (HPLC-UV). The HPLC-UV method has a limit of detection (LOD) of 0.36 mg/kg.[9] The sample preparation is time-consuming, as derivation of artemisinin is needed for proper UV detection. Because of the matrix complexity of soil

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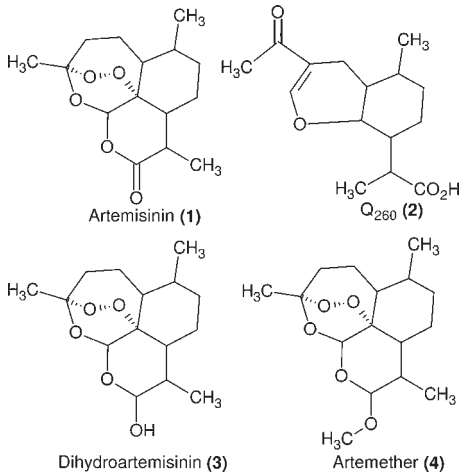

Figure 1. Structures of artemisinin (**1**); Q260 (**2**), the UV−visible compound that artemisinin is converted into after precolumn reaction; dihydroartemisinin (**3**); and artemether (**4**).

and plant extracts, the UV detection is less suitable for the quantitation of **1**; for example, soil components in organic soil may interfere with the analysis and quantitation of **1**. It is generally accepted that the use of structure-specific mass spectrometric detection is superior to UV detection for the quantitation of trace level analytes in complex matrices, in particular when derivatization is needed for UV detection. For example, a comparison study made for quantitation of ginsenosides, triterpenes from the herbal medicine ginseng,[14] revealed that quantification by liquid chromatography−tandem mass spectrometry (LC-MS/MS) was highly sensitive and specific as compared to HPLC-UV when applied to plant extracts. Transferring the analysis to LC-MS/MS could improve the performance of the methodology, since the high sensitivity and selectivity of mass spectrometry make it suitable for detection of low concentration compounds in complex environmental and biological matrixes. Several semiderivates of **1** can be considered as relevant when addressing the possible fate and impact of **1** in biological and environmental matrices. Hence, dihydroartemisinin (Figure 1, **3**), the sodium borohydride reduction product of **1**,[15] and artemether (Figure 1, **4**), the methyl ether of **3**,[16] both of which are more potent against malaria than **1**,[17] are included in the method development. It is not anticipated to find these derivatives in the *A. annua* fields, but including these compounds in the overall analysis makes the methodology more widely usable (i.e., in medical sciences). Details on the identification and quantitation of these more widely used drugs are also useful regarding contaminated water analysis, in terms of pharmaceutical residues. Furthermore, these compounds were evaluated for use as a suitable internal standard for **1** quantitation in environmental matrices.

The present study includes soil, plant, surface water, and groundwater matrices of particular importance for risk assessment and fate characterization of **1**. A sensitive, robust, and simple method using liquid LC-MS/MS was developed for the first time for the determination of **1** in soil. The method performance is demonstrated using both naturally contaminated soil samples and *A. annua* plant material sampled in fields used for production in Kenya. The area used for production of *A. annua* has increased dramatically in Kenya since the plant was introduced in the country in 2005 to approximately 5000 ha in 2010 [David Wainaina Wagacha, Director Botanical Extractions EPZ (BE-EPZ), East Africa, personal communication]. Because the release of **1** during cultivation may have hazardous effects on the soil fauna and yield of the following year's crop, it is highly relevant to measure the soil content and fate of the compound in these fields. The measured **1** concentrations were evaluated in relation to known ecotoxicological values, soil characteristics, and **1** content in the plants. The main purpose of the present study was to develop a method for quantitation of **1** in soil. To extend the versatility of the method, **3** and **4** were included in the method development, and the method versatility was evaluated by applying the method to plant extracts and spiked surface water and groundwater samples.

MATERIALS AND METHODS

Chemicals. *Chemicals and Reagents.* Artemisinin (**1**) [CAS,-63968-64-9; systematic 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, purity, 98%] and dihydroartemisnin (**3**) [CAS, 71939-50-9; systematic name, 3,12-epoxy-12*H*-pyrano(4,3-*j*)-1,2-benzodioxepin-10-ol, decahydro-3,6,9-trimethyl,(3*R*,5a*S*,6*R*,8a*S*,9*R*,10*S*,12*R*,12a*R*), a mixture of α and β isomers, purity, ≥97%] were purchased from Sigma-Aldrich (St. Louis, MO). Artemether (**4**) [CAS, 71963-77-4; systematic name, (3*R*,5a*S*,6*R*,8a*S*,9*R*,10*S*,12*R*,12a*R*)-decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano(4,3-*j*)-1,2-benzodioxepin, purity, ≥98%] was purchased from AvaChem Scientific LLC (San Antonio, TX).

Stock Solutions and Calibration Standards. Compounds **1**, **3**, and **4** stock solutions (3490, 4330, and 3980 μg/mL, respectively) were prepared in acetonitrile and stored in the dark at 5 °C. The stability for 1 month was validated (results not shown). The stock solutions were only removed from the refrigerator briefly and shaken when calibration standards were made. Calibration standards were prepared in acetonitrile in the concentration range of 0.008−2 μg/mL.

Sampling. *Locations in Kenya.* All soils were sampled in Kenya in July, 2009, with the exception of the Egerton sample, which was sampled in January, 2010. Four locations for sampling of *A. annua* var. *Artemis* were chosen in East Kenya [Naivasha (S 00°50.247 E 036°21.769/WGS84), Kirinyaga (S 00°41.080 E 037°21.837), Mweiga (S 00°19.314 E 036°52.220), and Kakuzi (S 00°58.141 E 007°002)], where the farmers have contracts with Botanical Extracts EPZ Limited (BE-EPZ) on their *A. annua* production. Seedlings were made available to the farmers from a nursery using seeds from Mediplant, Switzerland. This cultivar produces plants with high biomass and high **1** content with little variation, and the plants can grow under photoperiods of less than 13 h of light per day.[1] In West Kenya [Kajulu (S 00°01.547 E 034°47.781), Vihiga (N 00°04.255 E 034°40.661), and Butere (N 00°11.152 E 034°30.071)], the sampling sites were at smaller farmer's fields and gardens. The cultivation methods here were more primitive, and the farmers obtained their plants from an organization called Anamed.[18] The *A. annua* in this area is most likely the Anamed-A3 variety, a clone with a high leaf-to-stem dry matter ratio, known to be easily bred at latitudes from 0° to 20° N.[1] The Egerton site is located at Piave, Njoro, about 15 km from Egerton University, Kenya (S 0°19.44 E 35°56′38). The farmer planted 20 acres of *A. annua* in 2007 under the auspices of BE-EPZ. In 2008, he planted wheat in the entire plot. Because of crop failure that year, the farmer reverted to *A. annua* in 2009 but was unable to get enough seeds to plant the 20 acres. Consequently, some areas had *A. annua*, while the remaining sections were left fallow. Plant densities and cultivation practices are listed in Table 1.

Soil. A LC-MS/MS and solid-phase extraction (SPE) preconcentration and cleanup method was developed and applied to soils sampled in Kenya (Table 1). At seven locations, soil samples were taken in the

Table 1. Location, Soil Texture, and Characteristics of the Sampling Sites with Standard Deviations

					%							
sample location	plant density (plants/m^2)		type[a]	pH[b]	C[c]	N[d]	Al_{CBD}[e]	Fe_{CBD}[e]	clay[f] <2 μm	silt[f] 2−20 μm	fFine sand[f] 20−200 μm	coarse sand[f] 200−2000 μm
Soluti	0		ref. soil	4.8	1.0				24	3	42	31
Naivasha	8	monoculture, 4 years in a row	not cul.[e]	5.3	8.65 ± 0.08	0.71 ± 0.022	0.08 ± 0.002	0.55 ± 0.009	0.6	3.2	72	25
			0−2 cm	7.2	1.67 ± 0.013	0.16 ± 0.003	0.08 ± 0	0.63 ± 0.008	11	8.2	67	15
Kirinyaga	4	intercropping, melons and beans, first year	not cul.	5.1	2.48 ± 0.019	0.07 ± 0.004	4.26 ± 0.195	5.31 ± 0.272	40	33	25	1.0
			0−2 cm	5.3	2.24 ± 0.019	0.11 ± 0.015	4.13 ± 0.006	5.35 ± 0.008	38	35	26	0.9
Mweiga	8	monoculture, first year	not cul.	5.9	3.97 ± 0.035	0.29 ± 0.002	0.10 ± 0.002	0.83 ± 0.016	22	35	36	6.4
			0−2 cm	5.7	3.81 ± 0.025	0.29 ± 0.019	0.08 ± 0.029	0.62 ± 0.004	33	30	33	3.5
			2−5 cm	5.4	3.53 ± 0.034	0.30 ± 0.002	0.15 ± 0	1.18 ± 0.013	35	26	34	5.5
Kakuzi	5	self-seeded, 44 years in a row	not cul.	5.4	3.79 ± 0.042	0.26 ± 0.003	0.32 ± 0.001	3.50 ± 0.023	35	13	28	20
			0−2 cm	5.5	2.14 ± 0.012	0.09 ± 0.016	4.12 ± 0.003	4.87 ± 0.004	56	18	12	15
Kajulu	6	monoculture, first year	not cul.	6.1	1.09 ± 0.012	0.10 ± 0.004	0.11 ± 0.002	0.94 ± 0.008	28	8.0	30	34
			0−2 cm	5.9	1.83 ± 0.019	0.16 ± 0.008	0.14 ± 0.002	1.48 ± 0.02	28	29	30	13
Vihiga	4	intercropping with maize, 3 years in a row	0−2 cm	5.3	1.20 ± 0.018	0.11 ± 0.001	0.33 ± 0.007	1.86 ± 0.006	28	28	23	41
Butere	4	intercropping, 3 years in a row	not cul.	5.2	2.66 ± 0.002	0.18 ± 0.041	5.93 ± 0.003	5.25 ± 0.035	49	29	15	7.2
			0−2 cm	5.3	2.09 ± 0.021	0.06 ± 0.012	6.31 ± 0.76	5.41 ± 0.005	64	17	15	4.7
Egerton (A)[g]	8	monoculture, first year	not cul.	5.3	2.85 ± 0.009	0.24 ± 0.003	0.17 ± 0.002	2.67 ± 0.018	34	26	23	17
			0−5 cm	5.3	2.75 ± 0.013	0.22 ± 0.002	0.15 ± 0.001	2.48 ± 0.07	33	29	22	16
Egerton (F)[g]	0		0−5 cm	5.4	2.50 ± 0.006	0.21 ± 0.005	0.14 ± 0	2.45 ± 0.11	29	26	26	20

[a] Type of soil sample; not cul., margin soil not cultivated with *A. annua*, soil sampled in 0−2 cm depth or soil sampled in 2−5 cm depth. The Soluti soil sample is called the reference soil as *A. annua* has never been cultivated in that area. [b] pH measured in 0.01 M $CaCl_2$ once. [c] As determined as dry combustion in triplicates, except the samples from Egerton; they are determined in duplicates. [d] As determined by the Kjeldahl method in duplicates. [e] Citrate−bicarbonate−dithionite-extractable aluminum (Al) and iron (Fe) in duplicates. [f] The texture measurement is determined once. [g] Egerton (A) represents samples from the *A. annua* field, whereas Egerton (F) represents samples from a fallow field where *A. annua* was cultivated the previous year.

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upper 0−2 cm and in 2−5 cm depth using a scup. Samples were taken randomly from six different spots in each field and mixed 3 and 3 to two mixed samples. Margin soil samples were taken up to 8 m from the *A. annua* culture to obtain samples from soil where *A. annua* had never been cultivated but close enough to promote similar soil conditions. This criterion was fulfilled in most cases, except for the samples from the Naivasha location, where the margin soil differed remarkably from the *A.annua* sample. The soil texture and characteristic analysis was performed on a mix of all six soil samples from 0 to 2 cm from each location. From Mweiga soil, the texture and characteristics were also performed on a mixed soil sample from 2 to 5 cm. For two locations, Kakuzi and Butere, pairwise samplings of soil and plants from the same spots in young and old monocultures of *A. annua* were collected. Samples from Egerton were taken from both the *A. annua* area (marked A) and the fallow area, where *A. annua* had been cultivated the year before (marked F), using a soil auger at a depth of about 5 cm in January, 2010. A total of 10 samples were taken across each field in a zigzag manner. The samples were then mixed to obtain a composite sample for each part of the field. Margin soil samples not cultivated with *A. annua* from a nearby field with wild-growing plants other than *A. annua* were taken in a similar manner. A blank reference soil sample from Tanzania was also included in the method development. This soil was sampled in 1998 at Soluti in an earlier study. This soil represents sandy loam African soil and was sampled before *A. annua* was introduced to African countries. All soils were stored dry at 20 °C in darkness in paper bags until extraction.

Plants. Plant samples were taken randomly in the fields from both young and old leaves. For analysis, leaves from five different plants were mixed. All samples were air-dried in the shade and stored at 20 °C in the darkness in paper bags.

Surface Water and Groundwater. The surface water sample used in this pilot study was sampled at a lake included in the Danish National monitoring program (Utterslev Mose, Denmark, 55°43′19″, 12°31′6″ / WGS84) in April, 2011. The groundwater sample was collected from a Danish monitoring well at Tisvilde 10 m below surface in a glaciolacustrin aquifer layer.

Extraction. *Soil Extraction.* Extraction from soil was performed as described previously,[9] with the following modifications: 2.00 g of soil was weighed in 50 mL round-bottomed centrifuge glass, and 10 mL of 96% ethanol was added. These tubes were shaken upside down for 20−24 h followed by centrifugation for 10 min at a centrifuge force at 1.360*g*. Then, the supernatant was filtered through 20−25 mm Whatman 41 paper into 50 mL glass vials. The filter paper was rinsed once with 96% ethanol, and the extract was evaporated under nitrogen air flow until dryness. The dry extracts were stored at 5 °C until analysis.

Plant Material. Plant extraction was performed after a modified procedure initially based on extraction of **1** in chloroform.[19] In a preexperiment (results not shown), methanol was found to provide extraction efficiencies comparable to the original method, and for health and safety reasons, methanol was used in the present study. The modified extraction method was as follows: 0.10 g of dry crushed plant material was weighed out in a glass beaker. Three milliliters of methanol was added, and the mixture was stirred for 10 s. The samples were allowed to settle for 2 min, and then, the supernatant was filtered through 20−25 mm Whatman 41 paper into glass vials and evaporated to dryness under nitrogen air flow. The dry extracts were stored at 5 °C until analysis on HPLC-UV.

Method Evaluation in Surface Water and Groundwater Matrices. Recovery was evaluated using two volumes of water samples, 10 and 500 mL. The water samples were spiked with **1** in ethanol solution to obtain a final concentration of 7.5 ng/mL in the 10 mL samples and 0.2 ng/mL in the 500 mL samples. The volume of added ethanol solution corresponded to 5% of the total sample volume. The samples were spiked 2 h prior to SPE, where the aqueous samples were injected without further treatment.

SPE. *Preconcentration and Purification of Extracts.* The dry soil and plant extracts was redissolved in 0.5 mL of ethanol and diluted to 5.0% ethanol with demineralized water prior to SPE cleanup. Surface water and groundwater samples were applied directly to SPE. For SPE, Oasis MCX cartridges size 6 mL/150 mg (Waters, Milford, MA) were conditioned with 1 mL of methanol followed by 1 mL of demineralized water at 8.5 kPa vacuum. Then, the sample was loaded at 17 kPa vacuum. After extraction, the SPE cartridge was washed with 2 × 1 mL 2% v/v formic acid in water at 17 kPa vacuum followed by air drying at 34 kPa for 20 min. The analyte was eluted with three times 0.5 mL of acetonitrile. The three fractions were collected in a single 1.7 mL vial prior to LC-MS/MS analysis. Samples not analyzed directly were stored at 5 °C in darkness until analysis. Compound **4** was used as an internal standard and added to the eluted extract just before analysis.

HPLC-UV. *Quantitation of ArtO in Plant Material.* Because of the high concentration of **1** in the *A. annua* plant material, the HPLC-UV method was chosen for the initial quantitation of **1** in these. HPLC-UV-based determination of **1** was made using the method developed previously by Zhao and Zeng.[20] Compound **1** was converted to the strongly ultraviolet-absorbing compound Q260 (Figure 1, **2**) by a precolumn reaction. Dry plant extract was dissolved in 1 mL of 96% ethanol and treated with 4 mL of 0.2% NaOH (w/v) at 50 °C for 30 min in a water bath and cooled to room temperature. The solution was then acidified with 5 mL of 0.08 M acetic acid and filtered through a 0.45 μm Millipore filter (Millipore, Billerica, MA) before quantitation as **2** by HPLC-UV. The HPLC used was an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany). The samples were separated on a 250 mm × 4.6 mm i.d., 5 μm, Supelco Discovery Bio C_{18} wide pore column fitted with a 2 cm × 4.0 mm i.d., 5 μm guard column of the same material (Supelco Park, Bellefonte, PA). The mobile phase was a 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 rate was 1 mL/min, and the detection wavelength was set at 260 nm.

For comparison of HPLC-UV and LC-MS/MS method performance, two extra plant samples from Naivasha were extracted as described. Prior to the LC-MS/MS analysis, these extracts were redissolved in 0.5 mL of ethanol, cleaned up, and diluted 100 times in acetonitrile. The content was then quantitated by LC-MS/MS analysis.

LC-MS/MS. *Instrumentation.* The soil, surface water, groundwater, and plant samples were analyzed using a Waters Alliance 2695 LC system (Milford, MA) connected to a Quattro Ultima triple quadrupole mass spectrometer from Micromass (Manchester, United Kingdom). Quantitation based on peak area was performed in MassLynx version 4.0.

LC Separation. The liquid chromatography method was optimized based on Lindegardh et al.[12] The separation was performed using a 150 mm × 4.6 mm i.d., 5 μm Luna Phenyl-hexyl column (Phenomenex, Torrance, CA) at 50 °C. The mobile phase consisted of methanol: 10 mM ammonium acetate including 0.01 vol % acetic acid (70:30, v/v) (eluent A) and methanol. A gradient was used from 0 to 10 min 100% eluent A at a flow of 0.25 mL/min; 10−25 min 20% eluent A:80% methanol at a flow of 0.40 mL/min; and 25−30 min 100% eluent A at a flow of 0.25 mL/min. The sample injection volume was 10 μL. Retention times were 13.24 min for α-(**3**), 14.37 min for **1**, and 16.69 min for **4**, respectively. For comparison, the chromatography of the analytes was also evaluated using a modern 150 mm × 2.1 mm i.d., 2.6 μm porous shell particles based Kinetex C_{18} Ultrahigh performance column (Phenomenex).

MS/MS Conditions. Electrospray ionization (ESI) was performed in positive ionization mode. The optimum capillary and cone potentials were 3.45 kV and 15 V, respectively. Nitrogen was applied as the nebulization and desolvation gas (600 L/h, 250 °C). The source temperature was 110 °C, and a cone flow of 60 L/h nitrogen was used. Selective MS/MS detection of **1**, **3**, and **4** was achieved by measuring a

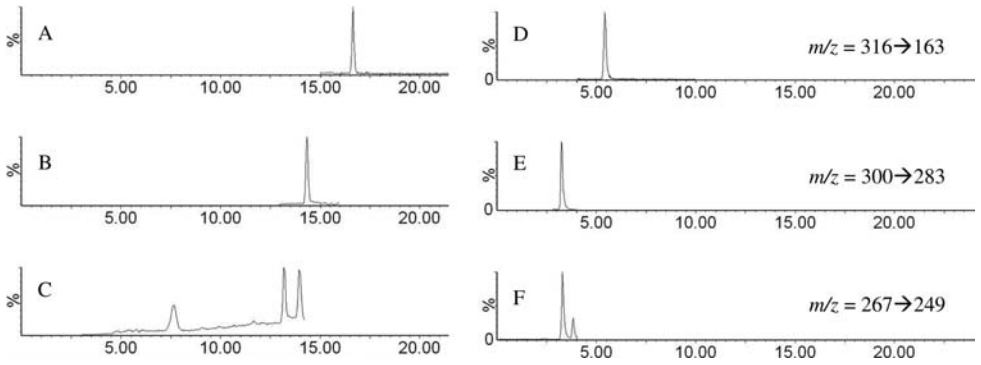

Figure 2. LC-MS/MS chromatogram (ion count of selected transitions as a function of time) for the two tested columns. A−C are chromatograms from a 150 mm × 4.6 mm Luna phenyl-hexyl column, 5 μm. D−F are chromatograms from a 150 mm × 2.1 mm Kinetex C_{18}, 2.6 μm Ultrahigh performance column. A−C are calibration standards of a mixture of (A) artemether (**4**) m/z 316 → m/z 163, (B) artemisinin (**1**) m/z 300 → m/z 283, and (C) dihydroartemisinin (**3**) m/z 267 → m/z 249 in acetonitrile at a flow rate of 0.250 mL/min in the soil matrix. Concentrations are (A) 0.163 μg/mL (rt = 16.64 min), (B) 0.165 μg/mL (rt = 14.34 min), and (C) 0.204 μg/mL (rt = 13.17 min). D−F are calibration standards of a mixture of (A) artemether (**4**) m/z 316 → m/z 163, (B) artemisinin (**1**) m/z 300 → m/z 283, and (C) dihydroartemisinin (**3**) m/z 267 → m/z 249 in acetonitrile at a flow rate of 0.1 mL/min. Concentrations are (D) 0.4 μg/mL (rt = 5.42 min), (E) 0.4 μg/mL (rt = 3.23 min), and (C) 4.4 μg/mL (rt = 3.28 min).

characteristic fragment of the parent ion at collision energy of 40 eV for **3**, 8 eV for the ammonium adduct of **1**, and 22 eV for the ammonium adduct of **4**. The ion traces were as follows: **3**, m/z 267 → m/z 249 measured from 3.00 to 14.20 min; **1**, $[M^+NH_4^+]$ m/z 300 → m/z 283 measured from 12.90 to 16.00 min; and **4**, $[M^+NH_4^+]$ m/z 316 → m/z 163 measured from 15.00 to 25 min (Figure 2).

Method Development and Optimization. *Cleanup Procedure Optimization—SPE.* The column material and solvent used for elution and percent dilution of the sample extracts prior to SPE were optimized. Retention of **1** on Oasis cartridges WCX, MAX, WAX, and MCX was tested, finding MCX to be the best sorbent material. The SPE method was developed following the Waters Oasis 2 × 4 method. Then, the optimum dilution grade of the raw ethanol soil extract at which **1** could be retained on the SPE column at the application step was found. This was tested at 96, 80, 60, 40, 20, 10, and 5 vol % ethanol extract. At 10% ethanol, a few percentage of **1** was still eluted during the application step, indicating that a lower content of ethanol was required for retention of the analytes during SPE application. At 5 vol %, no loss of analytes could be detected, and this ethanol content was implemented for the final method. Elution of the compound trapped on the SPE was evaluated using pure ethanol, ethyl-acetate, and acetonitrile as eluting solvents, resulting in 85, 8, and 93% recovery, respectively.

LC Separation. The LC part of the method was optimized based on a previous study by Lindegardh et al.[12] by testing different gradients. A gradient of 0 → 90, 80, and 70% methanol, respectively, was tested, showing that 0 → 80% methanol gave the best separation. Acetic acid gave a better adduct formation in the ionization than formic acid. Sequential MS optimization was performed for each analyte using syringe injection of the analyte in 70% methanol, 30% 10 mM ammonium acetate including 0.1% (v/v) acetic acid (eluent A). Initially, MS scan was used to optimize the capillary potential. Then, the cone potential was optimized followed by optimization of the collision energy. Performance was compared with quantification using same instrumentation but with a 150 mm × 2.1 mm i.d., 2.6 μm porous shell particles based Kinetex C_{18} Ultrahigh performance column (Phenomenex). This column was applied at 35 °C with a flow rate of 0.1 mL/min. The mobile phase was isocratic 60% eluent A. Nitrogen was applied as the nebulization and desolvation gas (550 L/h, 250 °C). The source temperature was 110 °C. A 55 L/h counter flow of cone gas was used. Retention times of 3.28, 3.23, and 5.42 min were obtained for **3**, **1**, and **4**, respectively, using the same ion traces as with the phenyl-hexyl column. In both systems, both α and β isomers of **3** were observed. From a previous study,[21] it is anticipated that the α isomer is the first eluted in a reverse phase LC system. As in ref 21, the α isomer was chosen for further quantification. In the Kinetex system, it was not possible to obtain complete separation of the α and β isomer peaks of **3** (Figure 2). It was also not possible to separate **1** and **3**, so these two analytes had to share scan time with reduction in the sensitivity as a result. For these reasons, no further method development with the Kinetex column was made, despite the short retention times. However, if analysis of **1** alone is the purpose, which could be the case in environmental matrices, the Kinetex column can be used with success.

Validation. To investigate possible matrix effects of having the three analytes in same mixture, calibration curves for all three analytes in single-compound solutions were compared to a calibration curve with all three analytes in a mixture. To estimate possible matrix effects of the soil extracts on ionization in the MS/MS method, concentrations ranging from 0.1 to 1 μg/mL of the analytes were added to soil extracts after the SPE purification. For a mixture of all three analytes, the linearity and range of the method were tested from 0.008 to 2 μg/mL. The within day variation (precision) was calculated as the standard deviation of six measurements of the same calibration standard containing a mix of all three analytes within the same day. The within day variation was calculated for three concentration levels close to the limit of quantification (LOQ). The between day variation (repeatability) was calculated as the standard deviation of measurements of the same calibration standard containing a mix of all three analytes over 3 days. The between day variation was calculated for three concentration levels close to the LOQ. Within day and between day variations are calculated on a concentration basis, so the daily fluctuations in the MS system are taken into account, as the purpose of this study was to develop a quantitative method. For all analytes, the LOD was calculated as $t_{(0.995,n=8)}$ × SD = 3.50 with eight measurements on a calibration standard with a concentration approximately five times higher than the expected LOD. The LOQ was calculated as 3 × LOD.

The recovery was evaluated in two steps: the SPE procedure alone by adding **1** in extracts without soil and the extraction procedure together with the SPE procedure, by spiking of soil samples. The recovery of spiked surface water and groundwater samples was also evaluated to test the versatility of the developed method.

Statistics. The measured soil concentrations of **1** were analyzed using a two-tailed t test with unequal variance. On the basis of soil concentration, the soils were divided into groups; i, the group where the concentrations in the *A. annua* culture were not significantly different

Table 2. Calibration Data, LODs, and LOQs Obtained for Artemisinin (1), Artemether (4), and Dihydroartemisinin (3)[a] Using the Developed LC-MS/MS Method

	calibration data			LOD[b]		LOQ[b]	
compd	linearity range (μg/mL)	equation	R^2	μg/mL	ng/g soil	μg/mL	ng/g soil
1	0.008−1	$y = 2 \times 10^6x + 28382$	0.997	0.030	25.5	0.090	76.5
4	0.008−1.2	$y = 177076x + 715.38$	0.9999	0.004		0.012	
3	0.008−2	$y = 133953x - 4.6609$	0.999	0.055		0.165	

[a] All data were obtained by analyzing standard mixtures prepared in acetonitrile. [b] LOD and LOQ are not calculated on soil basis for compounds **3** and **4**, as they are not analyzed in the soil environment.

from the concentration in the margin soils; ii, the group where the concentrations in the *A. annua* culture were significantly different from the one found in the corresponding margin soils and lower concentrations than the EC_{10} for lettuce growth;[9] and, finally, iii, the group where the concentrations in the *A. annua* culture were significantly different from the ones found in the corresponding margin soils and higher concentrations than the EC_{10} for lettuce growth. This EC_{10} was chosen because it is one of the few available toxicology data on **1** in soil and it is under the assumption that this result can be transferred from the laboratory to the field. The choice of an ecotoxicological value at the EC_{10} level is conservative. Evaluation of possible correlations between observed concentrations of **1** in soils and soil characteristics was made using multivariate analysis performed using Unscrambler (ver. 10.1, Camo Software Inc., Woodbridge, NJ).

RESULTS AND DISCUSSION

Validation. In the test for matrix effects or interference when having all three analytes in the mixture, the result was three parallel lines (peak area vs concentration) with the same slopes as for the three analytes independently. The slope for the three analytes in mixture was the same in matrix as without when plotted as peak area vs concentration; hence, there were no matrix effects to be encountered. The developed LC-MS/MS method had linearity in the range of 0.008−1 μg/mL. The slope of the standard curve decreased at concentrations higher than 1 μg/mL. The recovery of the extraction method for **1** in soil followed by SPE purification was 90%. The SPE method itself had a recovery of 94% when **1** in 5% aqueous solution was applied to the cartridges. The developed method has a LOD of 55, 30, and 4 ng/mL for **3**, **1**, and **4**, respectively. In Tables 2 and 3, the calibration data, LOD, and validation parameters for the method are summarized. The development of this method made it possible to use **4** as an internal standard in the environmental samples, as no interference between **4** and **1** was observed. Compound **4** was added as an internal standard to the combined eluate fraction from the SPE procedure, just before LC-MS/MS analysis of the samples. As compared to quantitation of **1** in soil samples by HPLC-UV, the developed method is more robust and 14 times more sensitive, the time-consuming derivatization step is omitted, and solvent consumption is reduced due to the lower flow used in the MS-based method. As compared to methods developed for quantitation of **1**, drugs in biological matrixes, where LOD in the range of 1 ng/mL can be obtained,[11−13] the present method had a LOD on the LC-MS/MS of 30 ng/mL, corresponding to 25.5 ng/g soil (Table 2). Thus, for the determination of **1** in complex soil extracts, the LOD was an order of magnitude higher in this method than previously reported for biological matrices. Still, the LOD and LOQ of the method developed were suitable for studies of **1** in the environment as soil concentrations could be quantitated in the range of relevance to ecotoxicological values such as EC_{10} for lettuce growth at 0.54 μg/g. Furthermore, the chromatography on the phenyl-hexyl column has been optimized to separate the analytes for any early eluting soil matrix elements (Figure 2A−C). This provides an option to lower the overall method LOD by increasing the mass of soil that is extracted. For the present study, this was not considered necessary.

Table 3. Within Day (n = 6) and between Day (n = 6) Variation of the LC-MS/MS Method

	μg/mL			
compd	calculated on concn level	within day[a]	calculated on concn level	between day[b]
1	0.82	0.09	0.66	0.013
	0.10	0.008	0.08	0.020
	0.05	0.007	0.02	0.006
4	0.65	0.070	0.65	0.008
	0.08	0.0004	0.08	0.005
	0.04	0.005	0.02	0.005
3	0.66	0.020	0.82	0.013
	0.08	0.002	0.10	0.020
	0.04	0.005	0.03	0.007

[a] Within day variation was calculated as the standard deviation on six measurements on the same mixture of standards in acetonitrile on the same day. Three standard mixtures with different concentration levels were used. [b] Between day variation was calculated as the standard deviation on six measurements on the same mixture of standards in acetonitrile over 3 days. Three standard mixtures with different concentration levels were used.

The SPE-LC-MS/MS method was also applied for analysis of plant extracts. In contrast to the soil samples, the plant extracts were diluted prior to clean up and analysis to be in the linear range of the method. The **1** content in the Naivasha plant sample was measured to be 88% of the content found with the HPLC-UV method when determined in diluted samples with LC-MS/MS. As no certified reference material is available, this comparison to traditional UV-based detection was chosen for evaluation of the LC-MS/MS method developed. The finding of a lower concentration in the plant material using the MS/MS based approach is supposed to be caused by matrix effects in the nonspecific UV-based quantification of the plant extracts, and for this reason, the MS/MS quantitation is preferred.

To further characterize the LC-MS/MS method developed, a preliminary test with spiked water matrices was made. In this study, **1** was cleaned up from spiked surface water and groundwater samples using the developed SPE-LC-MS/MS method.

Table 4. Artemisinin (1) Content in Kenyan Soil and Plant Samples

	concn of **1**			
	μg/kg DW soil[a]			mg/g DW plant[b]
location	soils not cultivated with *A. annua*	0−2 cm depth	2−5 cm depth	leaves
Soluti, reference soil	0	0	NA[c]	
Naivasha 1	39	317	212	1.48
Naivasha 2		170	152	
Kirinyaga 1	68	74	46	0.54
Kirinyaga 2		29	14	
Mweiga 1	3	1045	744	2.00
Mweiga 2		1135	2034	
Kakuzi 1, young	63	141	117	0.52
Kakuzi 2, old		455	56	
Kajulu 1	88	151	73	0.76
Kajulu 2		NA	74	
Vihiga 1	NA	54	NA	0.77
Vihiga 2		19	NA	
Butere 1, young	NA	0.4	NA	0.53
Butere 2, old		23	NA	0.97
Egerton (A)	125 ± 40	491 ± 184	NA	NA
Egerton (F)		99 ± 25	NA	

[a] Soil samples are true replicates as they are two composite soil samples from same field, except from Egerton, where two analyses are performed on the same composite sample. Those results are displayed with standard deviations. [b] Artemisinin (**1**) concentration in the plant material is two analyses on the same plant sample. [c] NA, samples were not available.

Results revealed that the method is suitable for these matrices as well, making the method of particular relevance for studies where the occurrence and fate of the compound is to be investigated in several matrices. The overall method recovery was evaluated at levels close to the LOQ using 500 mL surface water samples and 10 mL groundwater samples, demonstrating recoveries of 99 and 87% of **1**, respectively.

Content of 1 in Plant and Soil Samples from Kenyan *A. annua* Fields. The method performance was demonstrated using naturally contaminated soil samples and *A. annua* plant material sampled on location in fields used for production in Kenya (Table 4). All soils exposed to *A. annua* contained **1** in concentrations in the range of 10 to more than 1000 μg/kg dry weight (DW), and fields where *A. annua* had not been cultivated for very long seem to have a much lower content as found at Butere and Kakuzi, where samples were taken from both new and old areas of the cultivations. In many locations, **1** was also detected in soils not exposed to *A. annua* but at significantly lower levels than those observed in the *A. annua* crop field samples ($p = 0.0130$). To ensure the observed **1** concentrations in the unexposed samples were not false-positive response from the soil extracts, a soil sample from Tanzania (Soluti) was analyzed using the SPE-LC-MS/MS method as well. The Soluti site has never been exposed to *A. annua*. This sample gave no **1** response, and it was concluded that the findings in the marigin soils from Kenya were not false positives; that is, these border

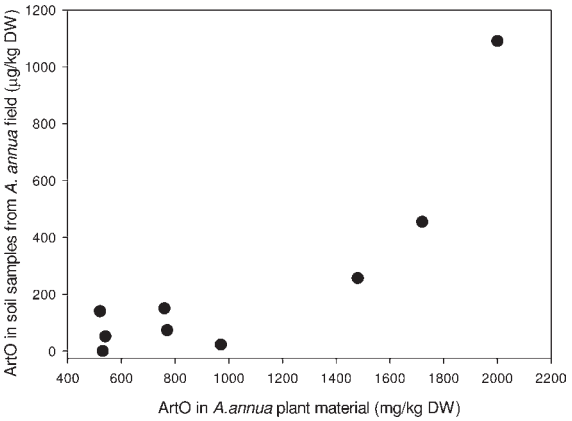

Figure 3. Compound **1** content in soil (μg/kg dry weight) as a function of **1** content in *A. annua* leaves (mg/kg dry weight).

area soils did contain **1**. This can be explained, for example, by previous cultivation of *A. annua*, wind transportation of leaf material, or transportation of released artemisinin by surface water runoff. The measured soil concentrations in 0−2 cm depth correspond to a soil concentration of up to 340 g/ha (soil density of 1.5 g/cm^3). The interfield variation was large and indicates spot wise release of **1** to the soil environment, for example, higher concentration at a spot where a dead leaf falls and decomposes.

As compared to the top layer margin soil, the soil samples from 0 to 2 cm could be divided into three groups based on the concentration of **1** and ecotoxicological data: i, concentrations in *A. annua* cultivation not significantly different (0.4−100 μg/kg); ii, concentrations in *A. annua* cultivation significantly different (100−500 μg/kg); and iii, *A. annua* cultivations with concentrations significantly different from margin soils and higher than the EC_{10} for lettuce growth (>500 μg/kg). Samples from Butere, Kirinyaga, and Vihiga were in group i ($p = 0.481$); Egerton, Kakuzi, Kajulu, and Naivasha were in group ii ($p = 0.008$); and finally, Mweiga was in group iii ($p = 7.8 \times 10^{-8}$). Similarly, the observed concentrations in the 2−5 cm soil samples could be divided into the same three groups. Kirinyaga, Kakuzi, and Kajulu were in group i, one Kakuzi sample and Naivasha were in group ii, whereas Mweiga again was in group iii. When all of the 2−5 cm samples were compared to the unexposed soils, there was no significant difference ($p = 0.176$), and the same was the case with group i ($p = 0.697$). Groups ii and iii from 2 to 5 cm differed significantly from the unexposed soils ($p = 0.046$ and $p = 0.005$). Plant density seemed to be playing a role, as all locations in group i only had a plant density of 4. Mweiga was the only location where the measured concentration of **1** could potentially affect other crops. The concentration of **1** found in Egerton is in the range where ecotoxicological effects may occur when evaluated using the EC_{10} value for lettuce growth. Considering persistence, it is noted that **1** is still measurable in soil 1 year after cultivation of *A. annua* as seen for Egerton (F).

Analyzing the data, a tendency could be identified (using linear as well as logistic correlation). A correlation between **1** content in leaves and in soil (linear, $r^2 = 0.73$) was observed (Figure 3). The more **1** produced by the plant, the more appears in the soil environment. This also explains why plant density is found to be important for concentration of **1** in soil, the higher plant density the higher concentration of **1** in the soil. At the two sites, Kakuzi and Butere, where both soil and plant samples were collected

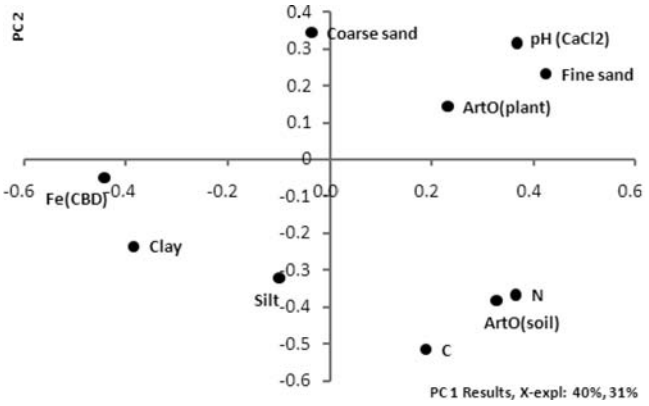

Figure 4. Principal component correlation of **1** contents in the analyzed Kenyan soils and selected soil characteristics.

from a young and old groups of plants of *A. annua*, it was observed that the older the plants, the more **1** they produce, and the more **1** is measurable in the soil below. For a deeper understanding of the tendency observed, in particular with respect to a logistic relationship between plant and soil content of **1**, knowledge about degradation rates in the different soils would be necessary. There was no correlation between sampling location (East or West Kenya) and observed **1** content in plants and soil.

A multivariate approach was used to analyze the data to identify possible relationships between **1** concentrations found and soil parameters (Figure 4). On the basis of data from the 16 Kenyan soils [Egerton (A), Egerton (F), and the margin soil from Egerton were identified as outliers and omitted from the analysis], a four principal component (PC) analysis could account for more than 95% of the variance in the data set. The loading plots of PC1 (40%) and PC2 (31%) demonstrate that high contents of **1** in soils coincide with high soil content of N, C, and a high soil pH. As seen in Figure 4, the possible correlation between the content of **1** in plant material and in soil was also indicated by the PC analysis. There was a tendency that the measurable content of **1** increases with soil organic matter. This could be caused by a lower degree of degradation of **1** sorbed to organic matter. In contrast, high contents of **1** seemed to be related to low content of Fe in the soils. PC1 indicated a relationship between low contents of **1** in soil and relatively high levels of texture component clay. This was not a simple relation though, as both silt and clay are in the same direction on PC2, but obviously, soil texture was of importance for the content of **1** in soil. Both silt and clay contain metal oxides on the surfaces,[22] and the PC1 texture correlation with content of **1** could be explained in that way. The proposed mechanism of **1** toward the malaria parasite is activation of the molecule by cleavage of the endoperoxide bridge. This process is catalyzed by iron II, originating from human heme degradation caused by the *Plasmodium* infection.[23] As suggested in previous degradation studies, it is possible that a similar mechanism can occur with metal oxides in soil.[9] Thus, the tendency that a low content of metal oxides in soil resulted in a higher concentration of **1** could possibly be caused by a slower degradation rate in these soils, but such a correlation between soil texture, content of metal oxides, and **1** has not been investigated in controlled experiments. The impact of soil texture and mineralogy to plant physiology of *A. annua* might also influence **1** in soils. To our knowledge, such a correlation has not yet been investigated. The main purpose with the present study was to develop an analytical method for quantitation of **1** in soil matrix; hence, the data set is not extensive enough to state a general tendency that a high content of organic matter together with a high content of metal oxides minimize the risk of a harmful concentration of **1** in soil. However, the possible correlation identified by the multivariate analysis should be investigated further in sorption and fate studies of this important medicinal compound in environmental matrices.

The developed SPE-LC-MS/MS method was proven suitable for determination of the concentration of **1** in soil, and the versatility of the method was demonstrated on surface water and groundwater samples, as well as in plant extracts. The developed method provides an analytical tool for monitoring **1**, **3**, and **4** in environmental matrices, and in cases where only **1** is investigated, a fast alternative to common C_{18} columns was identified based on a Kinetex C_{18} column. The results from the pilot studies demonstrated both the suitability of the method in different matrices and the need for further studies on the fate and impact of **1** on the soil environment.

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