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Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography–electrospray quadrupole time-of-flight tandem mass spectrometry

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Abstract

This study reports the development and validation of a rapid, sensitive and selective assay for the quantitation of artemisinin, arteannuin B, artemisitene and artemisinic acid in *Artemisia annua* L. by reversed phase high performance liquid chromatography (HPLC) electrospray (ESI) quadrupole time of flight (Q-TOF) tandem mass spectrometry (MS/MS). A recovery of >97% for all analytes was achieved by immersing one gram of fresh plant material in chloroform for 1 min. This result supports the hypothesis that artemisinin and some of its structural analogs present in the leaves *A. annua* L. are localized entirely in the subcuticular space of the glands on the surface of the leaves. We validated the use of this chloroform extract, without additional sample preparation steps, for quantitative Q-TOF MS/MS. No ion suppression (matrix effect) resulting from interference with other compounds was detected. For every concentration within the range of the standard curve (0.1 to 3.00 μg/ml), accuracy was between 85% and 115%. Within- and between-day variations for the analysis of *A. annua* L. samples were <20%.
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1. Introduction

Malaria is one of the world's most important parasitic diseases. There are at least 300 million acute cases of malaria each year globally, resulting in more than a million deaths [1,2]. Multi-drug resistance of the *Plasmodium* strains to the cheapest and most widely used antimalarials such as chloroquine, mefloquine and sulfadoxine-pyrimethamine is one of the biggest challenges in the fight against malaria [1].

Artemisia annua L. (sweet wormwood), a herb of the Asteraceae family has been used for centuries for the treatment of fever and malaria [3]. Artemisinin, an endoperoxide-containing sesquiterpene lactone, is the main component responsible for this therapeutic effect. Based on artemisinin, several semi-synthetic derivatives such as artemether, arteether and artesunate have been produced [3]. The WHO recommends that all countries experiencing resistance to conventional monotherapies should use combination therapies, preferably those containing artemisinin derivatives (ACTs—artemisinin-based combination therapies) [4,5].

As artemisinin cannot be synthesized chemically in an economically feasible way, *A. annua* is the only practical source of this valuable drug [6]. Unfortunately, *A. annua* contains only very small amounts of artemisinin ranging from 0.001% to 1.54% of dry weight [7]. Several research programs have been set up trying to increase the concentration of artemisinin in *A. annua* by optimizing the growing and harvesting conditions, by selecting high yielding cultivars or by creating transgenic plants [6,8]. To study the content of artemisinin and its biosynthetic precursors in plants, we developed a very simple extraction method followed by HPLC–ESI MS/MS.

Several other methods have been reported for the extraction, chromatography and detection of artemisinin and its structural analogs in *A. annua*. Liquid solvent extraction of dried plant

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material is currently the most commonly applied technique. Also more complicated extraction techniques such as super critical fluid extraction (SFE), pressurized solvent extraction (PSE) and microwave-assisted extraction (MAE) have been used. For the quantitation of artemisinin a large array of techniques have been developed including thin layer chromatography (TLC), high performance liquid chromatography with UV detection (HPLC-UV), HPLC with electrochemical detection (HPLC-ECD), HPLC with evaporative light scattering detector (HPLC-ELSD), gas chromatography with flame ionisation detector (GC-FID), GC coupled to mass spectrometry (GC–MS), GC coupled to tandem mass spectrometry (GC–MS/MS), supercritical fluid chromatography with FID (SFC-FID), ELSD (SFC-ELSD) or MS (SFC-MS) and capillary electrophoresis with UV detection (CE-UV). A review by Christen et al. [9] gives an excellent overview of these techniques and discusses some of them in more detail.

Some of these methods such as TLC, EC and UV-detection (artemisinin is UV-transparent therefore derivatisation is required) are time-consuming and not suited for routine analysis. More important is the fact that most of these methods lack specificity (TLC, UV-detection, FID, ECD, ELSD). As an *A. annua* plant extract may contain hundreds of components, some structural analogues of artemisinin, good specificity of the detector is essential.

The high sensitivity and selectivity of MS and certainly MS/MS present a major advantage for the detection of specific components in plant extracts. Several GC–MS [10,11], HPLC-MS [12–15] and HPLC-MS/MS [16] methods have been developed to analyze artemisinin and its derivatives in blood, plasma or serum. For analysis of *A. annua* extracts a SFC-MS method has been reported [17].

To our knowledge, we report the first MS/MS method developed to analyze artemisinin and its biosynthetic precursors in *A. annua*. The main advantages of our method are not only the excellent specificity but also the extremely short and efficient sample preparation.

2. Experimental

2.1. Chemicals

Pure reference standard of artemisinin, 98% was obtained from Sigma–Aldrich (Bornem, Belgium). The other reference standards arteannuin B, artemisitene and artemisinic acid were kindly provided by the Walter Reed Army Institute of Research (Washington, USA). The internal standard (I.S.) β-artemether was a gift from Arenco Pharmaceutica N.V. (Geel, Belgium).

LC–MS grade absolute methanol was obtained from Biosolve (Valkenswaard, the Netherlands). Analytical grade chloroform was obtained from Acros (Geel, Belgium). Analytical grade ammonium acetate, analytical grade sodium acetate and acetic acid (99.8%) were obtained from Sigma–Aldrich (Bornem, Belgium). Purified water of 18.2 MΩ/cm was obtained from a Milli-Q system (Millipore, Belgium).

2.2. Artemisia annua L. plants

The plants were grown under controlled conditions (21 °C; 12 h day/12 h night regime). Seeds were kindly provided by the National Botanic Garden of Belgium (Meise, Belgium).

2.3. Analytical standards

Individual stock solutions (1 mg/ml) of artemisinin, arteannuin B, artemisitene, artemisinic acid and internal standard β-artemether were prepared by accurately weighing required amounts into separate volumetric flasks and dissolving in appropriate volumes of methanol. Analytical standards were prepared as a mixture of each analyte (0.1 μg/ml to 3 μg/ml each) and the internal standard (0.4 μg/ml) by serial dilution of stock solutions in methanol–1 mM ammonium acetate buffer adjusted to pH 5 with acetic acid (50/50, v/v).

2.4. Sample preparation

Extraction was performed by immersing one gram of plant material in 6 ml chloroform for 1 min. An aliquot of 10 μl of this extract was then dissolved in 1 ml methanol–1 mM ammonium acetate buffer adjusted to pH 5 with acetic acid (50/50, v/v) containing 0.4 μg/ml of the I.S. β-artemether. This procedure was carried out on the plants of interest: *Artemisia annua* L. (Asteraceae) and the negative controls *Artemisia Absinthium* L. (Asteraceae), *Mentha spicata* L. (Lamiaceae) and *Mentha piperita* L. (Lamiaceae).

2.5. Liquid chromatography

A Waters Alliance 2695 HPLC system was used to deliver the mobile phase [pump A, 1 mM ammonium acetate buffer adjusted to pH 5 with acetic acid; pump B, 100% methanol] for gradient elution at a flow rate of 0.2 ml/min. The initial composition of 50:50 was maintained for 1 min; next the methanol content was increased linearly to 80% over a period of 6 min and maintained for 18 min. Re-equilibration time was 10 min between runs. The sample injection volume was 100 μl for all samples. Chromatographic separations were achieved on an Alltech Ultrasphere C_{18} IP 5 μm column (150 mm × 2.1 mm) protected by a Waters XTerra MS C_{18} 5 μm guard column (10 × 2.1). A LC Packings ACUrate ICP-04-20 post-column splitter was used to divert one-fourth of the effluent into the electrospray LC–MS interface.

2.6. Q-TOF mass spectrometry

Mass spectrometric detection was performed on a Q-TOF Ultima mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray source operating in positive mode (ESP+). The ESI capillary voltage was set at 2.7 kV. The source and desolvation temperatures were optimized at 130 and 300 °C, respectively. Nitrogen was used as desolvation gas with a flow rate of 500 l/h. MS/MS analysis was performed using argon (0.9 bar) as the collision gas. An MS/MS method was used to quantify artemisinin (m/z 283 → 219 + 229 + 247 + 265),

arteannuin B (m/z 249 → 185 + 189 + 203 + 231), artemisitene (m/z 281 → 217 + 227 + 245 + 263), artemisinic acid (m/z 252 → 189 + 199 + 217) and β-artemether (m/z 316 → 267 + 284 + 307). Cone voltage had an optimum at 40 V for all components. The collision energy was optimized at 7 eV for artemisinin, 10 eV for arteannuin B, 7 eV for artemisitene, 11 eV for artemisinic acid and 7 eV for β-artemether. Data acquisition and analysis were carried out using Masslynx version 4.0. software. Analytical standard curves (second-order polynomic regression) were calculated using analyte to I.S. peak area ratios. The concentrations of the respective analytes in test samples are interpolated from the standard curves using the analyte to I.S. peak area ratios from the test samples.

3. Results and discussion

3.1. Sample preparation

Most plant extraction methods start with lyophilisation or drying of the plant material, followed by extraction with an organic solvent such as hexane or toluene [9]. As all cells are disrupted by these extraction methods, all soluble components are extracted from the plant. These extracts contain a massive amount of components (e.g. chlorophyll) interfering with HPLC (clogging) and MS (matrix effect). Additional sample preparation has to be performed prior to HPLC–MS/MS. Unfortunately these additional steps (solid phase extraction, filtering, evaporation steps) are not only time-consuming, but are also a possible source of variations in recovery.

In the specific case of the extraction of artemisinin and its bioprecursors from *A. annua*, these problems can be avoided. Duke et al. [18] reported that a 5 s dip in chloroform extracted 97% of the artemisinin and 100% of artemisitene from *A. annua*. In the report by Duke et al., quantitation was performed by HPLC-UV after derivatisation. Light microscopy and transmission electron microscopy revealed that the 5 s dip results in collapse of the subcuticular cavity of the glands on the leaf surface but did not disrupt cell membranes. An *A. annua* biotype without glands contained neither artemisinin nor artemisitene [18]. These results indicate that artemisinin and artemisitene present in foliar tissue are localized entirely in the subcuticular space of glands of *A. annua*.

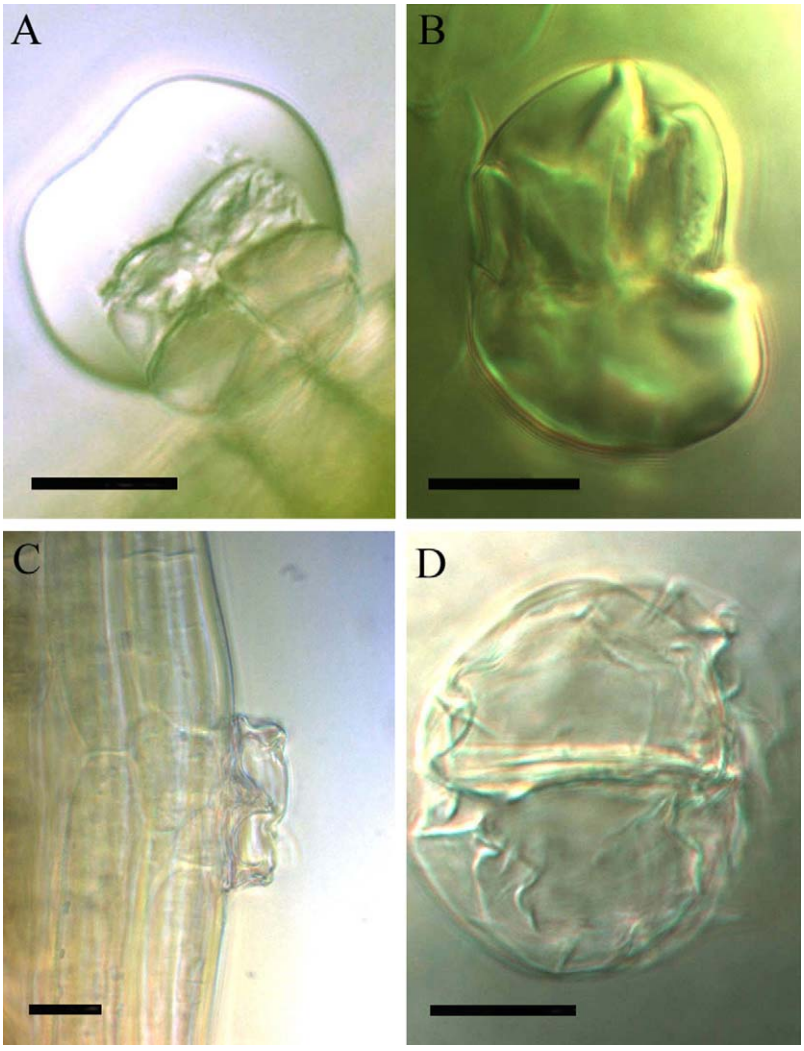

Fig. 1. Picture of a glandular trichome on a leaf of *A. annua* L. before (A and B) and after (C and D) chloroform extraction. The cuticle is crumpled after chloroform extraction. The epidermal cells are unaffected by this treatment. Black bar is 10 μm.

We hypothesized that this chloroform extract can be analyzed on a mass spectrometer without additional sample preparation steps as it contains only a very small quantity of interfering components (e.g. chlorophyll) compared to plant extracts where the plant material is lyophilized, dried or grinded.

3.2. Extraction time

We decided to prolong the extraction time as long as possible to break open as much glands as possible without introducing interfering compounds. After an extraction time of 1 min, chlorophyll starts to be released into the chloroform, indicating that cells with interfering compounds begin to break open. Fig. 1 shows a picture of glandular trichomes before and after a 1 min chloroform extraction. The cuticle is crumpled after chloroform extraction. The epidermal cells are unaffected by the treatment. The extraction time of 1 min was validated during the recovery studies.

3.3. Recovery

Two different experiments were done to asses the recovery. In a first experiment, 15 equal samples of one gram fresh *A. annua* leaves were prepared, 5 of which were spiked with 60 μl of a 10 mg/ml methanol solution of each analyte. Immediately after evaporation of the methanol, all 15 samples were analyzed. The recoveries of the 5 spiked samples were calculated as the ratio between the measured quantity and the spiked quantity increased with the mean quantity of the analytes in the 10 unspiked samples. Table 1 shows the mean of the recoveries for the different analytes (>97% for each analyte).

This very high recovery (>97%) of the spiked amounts does not imply a high recovery of the amounts present in the plant. The recovery of the amounts present in the plant with our method, cannot be measured directly. Therefore, we estimated this recovery in a second experiment by comparing the recovery achieved with a previous described extraction method [19] before and after our one-minute chloroform extraction. Six equal samples of one gram fresh leaf material were prepared. Three of them were extracted following a previously described extraction method [19]. Briefly, this method consists of an extraction with 2 × 3 ml toluene after lyophilisation and pulverization of the plant material followed by a normal-phase Silica gel solid-phase extraction (SPE). An aliquot of 1 ml of plant extract was passed through the 500 mg Silica gel column, followed by washing with 2 ml petroleum ether–diethyl ether (9:1) and elution with 2 × 0.5 ml acetonitrile. The eluate was evaporated to dryness under N_2 and reconstituted in 1 ml methanol–ammonium acetate buffer (50/50, v/v) for further analysis. Note that compared to our method, the analytes are a 100 fold more concentrated by this SPE. The other three samples were subjected to exactly the same extraction protocol, but after they were first extracted by our method (1 min chloroform extraction). Table 2 gives and overview of the results. The amount of artemisinin, arteannuin B and artemisinic acid found in the plant material after chloroform treatment was less than 3% compared to the amount found in the three non-pretreated samples. This experiment shows that >97% of artemisinin, arteannuin B, and artemisinic acid is extracted by a 1 min dip in chloroform. As a 1 min dip in chloroform is the only sample preparation step in our method, we conclude that a recovery of > 97% can be achieved by our method. For artemisitene, the results are less conclusive as the measured quantity after chloroform extraction falls below the lower limit of quantitation (LLOQ). Nevertheless, the experiment gives a good indication of a high recovery of artemisitene.

3.4. Chromatography

During flow injection analysis on the Q-TOF MS, $[M + Na]^+$ adducts were found to be far more intense than $[M + H]^+$ or $[M + NH4]^+$ adducts. At first we tried to intensify the $[M + Na]^+$ adducts, reducing other adducts by using sodium

Table 1
Recovery from spiked samples

Spiked quantities (μg/ml)	Arteannuin B	Artemisitene	Artemisinin	Artemisinic acid
Mean quantity unspiked samples[a]	0.37	0.06	0.17	0.42
Spiked quantity[b]	1.00	1.00	1.00	1.00
Total quantity in spiked samples[c]	1.37	1.06	1.17	1.42
Recovered quantities (μg/ml)	Arteannuin B	Artemisitene	Artemisinin	Artemisinic acid
Spiked sample 1[d]	1.47 (107.39%)	1.13 (106.30%)	1.24 (106.12%)	1.44 (100.87%)
Spiked sample 2[d]	1.27 (93.01%)	0.97 (91.27%)	1.06 (90.23%)	1.26 (88.49%)
Spiked sample 3[d]	1.51 (110.53%)	1.22 (115.15%)	1.28 (109.37%)	1.52 (106.66%)
Spiked sample 4[d]	1.14 (83.47%)	0.89 (83.70%)	0.96 (81.59%)	1.19 (83.48%)
Spiked sample 5[d]	1.31 (95.74%)	1.01 (95.14%)	1.15 (98.55%)	1.56 (109.45%)
Mean spiked samples (μg/ml) [e]	1.34 (98.03%)	1.04 (98.31%)	1.14 (97.17%)	1.39 (97.79%)
Standard deviation (μg/ml)	0.15 (11.03%)	0.13 (12.46%)	0.13 (11.42%)	0.16 (11.35%)

[a]Fifteen equal samples of one gram fresh *A. annua* leaves were prepared. Ten samples were not spiked and analyzed. [b]Five samples were spiked with each analyte. [c]The total quantity of the analytes present in the spiked samples was calculated as the sum of the spiked quantity and the mean quantity of the analytes in the 10 unspiked samples. [d]The spiked samples were analyzed and the individual. [e]Mean absolute recoveries (% recovery between brackets) were calculated. Quantities are presented as the concentration after sample preparation (multiply by 600 to obtain quantities in μg analyte/g fresh plant material).

Table 2
Recovery with chloroform extraction of *Artemisia annua* leaves

	Arteannuin B	Artemisitene	Artemisinin	Artem. acid
Mean quantity WITHOUT preceding chloroform extraction (μg/ml)[a]	58.64	0.28	24.17	72.07
Mean quantity AFTER preceding chloroform extraction (μg/ml)[b]	3.60	0.02 (< LLOQ)	1.58	5.53
Quantity not extracted by preceding chloroform extraction (%)	6.14	6.80	6.55	7.68
Residual chloroform in samples after chloroform extraction[c] (%)	>5	>5	>5	>5
Recovery (%)	>98.86	>98.20	>98.45	>97.32

[a]Six equal samples of one gram fresh leaf material were prepared. Three of them were extracted following a previously described extraction method [19] which uses extraction with toluene after lyophilisation and pulverization of the plant material. [b]The other three samples were extracted in exactly the same way but after they were first extracted for one min with chloroform. [c]The percentage of the chloroform which sticks to the plant material after chloroform extraction (accounting for a part of the not-extracted percentage) was gravimetrically determined. Quantities are presented as the concentration after sample preparation (multiply by 6 to obtain quantities in μg analyte/g fresh plant material).

acetate buffer and performing analysis on the $[M+Na]^+$ adducts. This approach was abandoned due to variation caused by build up of sodium acetate deposits on the ion sampling cone of the mass spectrometer. Finally we decided to use ammonium acetate buffer and to perform MS/MS analysis on the $[M+H]^+$ (artemisinin, artemisitene, arteannuin B) or $[M+NH4]^+$ (artemisinic acid, artemether) adducts.

A total of 3 isocratic and 25 gradient elutions were compared, testing varying methanol–buffer ratios and testing varying gradient speeds. The method with the highest peak resolution was chosen. The reproducibility of the retention times was very dependent on the buffer concentration. Increasing the buffer concentration from 0.1 mM to 1 mM greatly enhanced the reproducibility of the retention times, resulting in a variation of less than 25 s. By varying the pH of the ammonium acetate buffer, the retention time of artemisinic acid can be influenced. Peak resolution was optimal at pH 5. Using final conditions, all analytes were separated from each other with peak resolutions from 1.0 to 2.4 (Fig. 2).

The column and guard column were stable for at least 1000 injections. No signs of column deterioration have been detected yet.

3.5. Specificity

In contrast with $[M+Na]^+$ adducts, the $[M+H]^+$ and $[M+NH4]^+$ adducts were easily fragmented with low collision energies. Fig. 3 shows the fragmentation spectrum of artemisinin at an optimal collision energy of only 7 eV. Between 3 and 4 fragments were chosen to be monitored for each analyte. Using the sum of several fragments for MS/MS quantitation, has the advantage of increased signal strength and enhanced signal stability, but the disadvantage of lower specificity. As fragments with higher *m/z* values tend to be more specific, fragments with the highest *m/z* values were selected.

To check the specificity of the method, chloroform extracts of *Mentha piperita*, *Mentha spicata* and *Artemisia absinthium* were analyzed. These three plant species also have epidermal glands

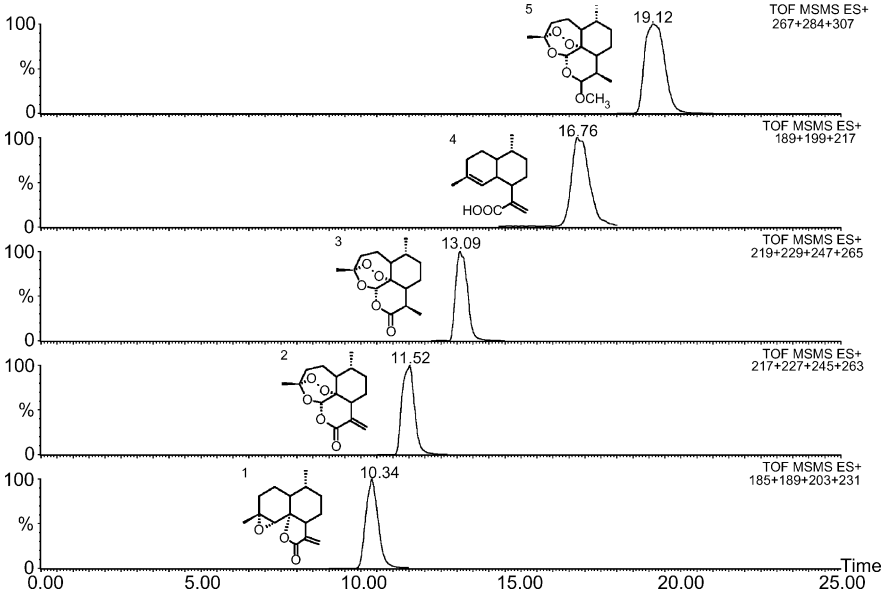

Fig. 2. Chromatogram with retention times and chemical structures of (1) arteannuin B, (2) artemisitene, (3) artemisinin, (4) artemisinic acid and (5) the internal standard artemether. This chromatogram is the result of the analysis by electrospray QTOF-MS/MS of an analytical standard containing 1.2 μg/ml of each analyte and 0.4 μg/ml IS.

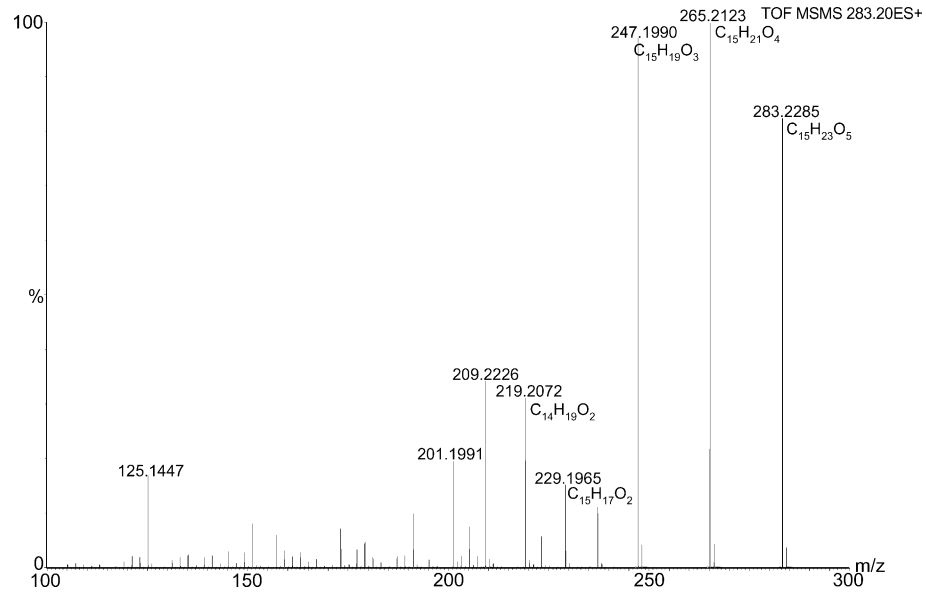

Fig. 3. This MS/MS spectrum shows the fragmentation of artemisinin (*m/z* 283) with the optimal collision energy of 7 eV. The MS/MS signal is calculated as the sum of the fragments with *m/z* 219, 229, 247 and 265. The molecular formulas show that the fragments are mainly formed by dehydration of their parent ion.

on their leaves, but are not reported to produce artemisinin. In these control extracts, no MS/MS signal could be detected for the components of interest.

3.6. Ion suppression (matrix effect)

To check for interferences from other compounds by ion suppression (matrix effect) a standard curve obtained from standards prepared in mobile phase was compared to a standard curve obtained from standards made in mobile phase spiked with matrix (10 μl chloroform extract of *Mentha piperita* /ml). HPLC-MS/MS analysis (3 measurements for each sample) of these standards, resulted in almost identical measurements for the spiked and the non-spiked standards. The statistical method of Bland et al. [20] was used for assessing the agreement between the two methods. The *p*-values for the *t*-test with the nulhypothesis that the mean of the differences between both methods is equal to zero, were 0.53, 0.26, 0.67 and 0.74 for arteannuin B, artemisinin, artemisitene and artemisinic acid, respectively. No significant difference could be found for any of the analytes, meaning that no ion suppression could be detected. For this reason, in the final method, standards were not spiked with chloroform extract to include matrix.

3.7. Accuracy, precision, limit of detection (LOD) and lower limit of quantitation (LLOQ)

The definitions for accuracy, precision, LOD and LLOQ were adopted from the FDA guidelines for bioanalytical method validation [21]. The LOD was defined as the lowest observable peak response for an analyte above the background noise, 3 times the system noise in the matrix. The LLOQ was defined as the lowest concentration for an analyte with a response signal 5 times the system noise in the matrix, a precision of 20% and an accuracy of 80–120%. Within-day accuracy and precision were calculated with three determinations on one day. Between-day accuracy and precision were calculated from 7 determinations on 3 days spanning a two week period. Accuracy and precision were calculated for each of 7 spiked concentrations (0.1; 0.2; 0.4; 0.8; 1.2; 2.0 and 3.0 μg/ml) within the range of the standard curve. Within the range of the standard curve, coefficient of variation (CV%) was <15% and accuracy was between 85% and 115% for all analytes and all 7 spiked concentrations (Table 3).

Within- and between-day variation was also calculated for unspiked *A. annua* samples. Twenty equal samples of one gram fresh leaf material were prepared and kept between 4 °C to 8 °C until extraction. Ten of these samples were extracted with chloroform on day 1, five on day 2 and again five on day 3. The extracts were stored at −20 °C until HPLC-MS/MS analysis. The first 10 extracts (extraction on day one) were analyzed in one day allowing calculation of within-day variation. The other 10 extracts were analyzed on two different days spanning a two week period. All 20 independent samples were used to calculate the between-day variation. The within- and between-day variation of the complete procedure (extraction and quantitation by MS/MS) is <20% (Table 3) for all analytes except for artemisitene for which the amount present in the unspiked samples was below the LLOQ. The variation for the unspiked samples is higher than for the spiked samples. A possible reason is variation in the release of the analytes out of the glandular trichomes. Another possibility is an actual variation in the 20 samples as the leaves for these samples were collected from three different plants.

3.8. Dynamic range and polynomial regression

Based on the LLOQ and dynamic range of the MS/MS signal, standard curves were established from 0.1 to 3.00 μg/ml

Table 3
Accuracy, precision, LOD and LLOQ

	Arteannuin B		Artemisitene		Artemisinin		Artemisinic acid	
	Within	Between	Within	Between	Within	Between	Within	Between
LOD (μg/ml)	0.001 (S/N: 3)		0.0005 (S/N: 5)		0.0001 (S/N: 9)		0.04 (S/N: 4)	
LLOQ (μg/ml)	0.1 (S/N: 86)		0.1 (S/N: 52)		0.1 (S/N: 22)		0.1 (S/N: 5)	
Accuracy at LLOQ (%)	108 ± 9	111 ± 8	107 ± 13	111 ± 8	114 ± 10	112 ± 6	104 ± 7	108 ± 8
CV% at LLOQ (%)	8.5	7.5	12.6	6.7	8.4	5.1	6.6	7.1
Accuracy (0.1–3 μg/ml spiked) (%)	94–108	94–111	89–107	85–110	90–113	85–111	97–104	96–107
CV% (0.1–3 μg/ml spiked) (%)	1.0–8.5	0.9–7.5	2.0–12.5	1.3–8.5	1.1–13.9	1.3–10.3	0.8–7.1	0.9–7.1
Mean unspiked extracts (μg/ml)	0.36	0.33	0.07	0.08	0.16	0.15	0.39	0.36
St. dev. unspiked extracts (μg/ml)	0.05	0.06	0.02	0.02	0.03	0.03	0.06	0.07
CV% unspiked extracts (%)	14	19	24	22	15	19	17	20

LOD and LLOQ are presented with peak-to-peak signal-to-noise ratio. Within- and between-day accuracy and precision are presented at LLOQ and for 7 spiked concentrations (0.1; 0.2; 0.4; 0.8; 1.2; 2.0 and 3.0 μg/ml) within the range of the standard curve. Within- and between-day variation was also calculated for 20 unspiked *A. annua* samples. Quantities are presented as the concentration after sample preparation.

for artemisinin, arteannuin B, artemisitene and artemisinic acid. Several regression models were evaluated to establish these curves. For the range of 0.1–3 μg/ml, a best-fitted second-order polynomial regression ($y = Ax^2 + Bx + C$) described the measurements of the analytical standards at best (typically $R^2 > 0.99$). Limiting the range to 0.1–0.8 μg/ml, a linear regression ($y = Ax + B$) would also be acceptable with $R^2 > 0.99$, but still a second-order polynomial regression describes this range better with $R^2 > 0.999$. In practice, the use of a second-order polynomial regression not only extended the useful dynamic range, but also reduced the between-day variation.

The range of the standard curves may not extend high enough to analyze high yielding plants [7]. Dilutions can be made from the extracts of these plants. To check if these dilutions do not present any ill effects, a sample of one gram fresh leaf material from *A. annua* was spiked with 16 mg of artemisinin in a methanol solution. After evaporation of the methanol, the artemisinin was extracted and prepared with the standard sample preparation procedure. Immediately before HPLC–MS/MS analysis, the sample was diluted 16 fold with methanol–ammonium acetate buffer (50/50, v/v) containing 0.4 μg/ml of the internal standard. The diluted sample was measured 3 times; recovery was 100.0% ± 8.4%.

4. Conclusions

This study reports the development and validation of a rapid, sensitive and selective assay for the quantitation of artemisinin, arteannuin B, artemisitene and artemisinic acid in *A. annua* L. by reversed phase HPLC ESI Q-TOF MS/MS. An absolute recovery of >97% was achieved by immersing one gram of plant material in chloroform for 1 min. This result supports the hypothesis that artemisinin and some of its structural analogs present in the leaves of *A. annua* L. are localized entirely in the subcuticular space of the glands on the surface of the leaves. We validated the use of this chloroform extract for quantitative MS/MS without additional sample preparation steps. No ion suppression (matrix effect) resulting from interference with other compounds was detected. To check the specificity of the method, chloroform extracts of *Mentha piperita*, *Mentha spicata* and *Artemisia absinthium* were analyzed. These three plants also have epidermal glands on the leaves, but do not synthesize artemisinin. No signal for the components of interest was detected in these control extracts. With a LOD of at least 0.04 μg/ml, a LLOQ of 0.10 μg/ml and a dynamic range from 0.10 to 3.00 μg/ml for each analyte, the method has enough sensitivity and flexibility to measure low and high yielding cultivars. For every concentration within the range of the standard curve (0.1 to 3.00 μg/ml), accuracy was between 85% and 115%. Within- and between-day variations for the analysis of unspiked *A. annua* L. samples were <20%.

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