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Short communication

Determination of artemisinin and its analogs in *Artemisia annua* extracts by capillary electrophoresis – Mass spectrometry

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ABSTRACT

The applicability of micellar electrokinetic capillary chromatography (MEKC) with mass spectrometric detection for the determination of artemisinin and its analogs (e.g. ascaridole, artemisia ketone, casticin, deoxyartemisinin, arteannuic acid, artemetin, dihydroartemisinic acid) was studied. 40 mM ammonium perfluorooctanoate (pH 9.5) with 2% isopropanol (IPA) was used as background electrolyte (BGE) and the sheath liquid was 50 % (v/v) IPA:water containing 0.1 % formic acid. Separation was performed in a bare fused silica capillary. Artemisinin was detected at 283.1545 *m/z* as $[M+H]^+$ ion. For artemisinin the linear range was found to be 0.6 μg/mL - 60 μg/mL and the limit of detection was 0.18 μg/mL. The RSD% values were 2.6 % for migration times and 4.8 % for peak areas (N = 6). In the ethanolic extracts of *Artemisia annua* leaves, in addition to artemisinin, a large number of other organic components could be separated and determined. MEKC-MS revealed the existence of diastereomers of several compounds (artemisinin, deoxyartemisinin, dihydroartemisinic acid) in the plant extracts.

1. Introduction

Malaria is a life-threatening disease affecting millions of people worldwide, with the African region having a disproportionately high share of cases. The emergence of drug-resistant parasite strains to existing antimalarials undermined the efforts of complete eradication [1]. Triggered by the urgent need for effective treatment, Tu Youyou and coworkers discovered antimalarial activity in the extracts of *Artemisia vulgaris* L. (Mugwort) in the 1970s. The component responsible for the therapeutic effect was identified to be artemisinin [2]. Artemisinin is a sesquiterpene lactone having an endoperoxide group, to which the antimalarial activity can be attributed, through the formation of free radicals [3]. Artemisinin derivatives, such as artesunate, dihydroartemisinin, artemether and arteether also play an important role in fighting off the infection. This revelation paved the way for the introduction of artemisinin-based combination therapies, saving the lives of many. In addition to being a potent antimalarial drug, there have been indications of artemisinins possessing anti-inflammatory and anticancer properties [4].

Owing to the great contribution of artemisinins to increased survival rates, there has been a considerable need for developing and applying reliable analytical methodologies to carry out pharmacokinetic studies and to monitor drug purity. The sensitive determination of artemisinin and its analogs however, pose quite a challenge due to their low concentration, thermolability and the absence of ultraviolet (UV), fluorescent chromophores or functional groups suitable for quantitative derivatization. Despite the difficulties, numerous methods have been proposed [5], including thin layer chromatography (TLC) [6], gas chromatography (GC) [7,8], nuclear magnetic resonance (NMR) [9], enzyme-linked immunosorbent assay (ELISA) [10], high-performance liquid chromatography (HPLC) with reductive electrochemical [11], UV [12] and mass spectrometric (MS) [13–16] detection. There have also been reports on capillary electrophoretic (CE) techniques, mainly for the quantification of the easily ionizable artesunate analog [17–19]. From the conventional CE point of view, artemisinin suffers from two important character flaws: the compound is i) neutral and ii) has low UV absorption. Automated derivatization was made possible by coupling CE with flow injection (FI) systems through a split-flow interface, enabling the conversion of artemisinin and the sensitive determination of the obtained chromophoric compound in a true, on-line fashion [20,21]. Due to the low UV absorption of artemisinin, CE with indirect UV detection would be an obvious choice; however, this has not been extensively researched probably

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because of concerns over sensitivity. To the best of our knowledge, no study has been proposed on the utilization of CE—MS for the analysis of artemisinin.

The separation of neutral components can be carried out using micellar electrokinetic chromatography (MEKC). In MEKC mode, the BGE contains a pseudostationary phase consisting of micelles, with which the neutral compounds interact, depending on their hydrophobicity. However, surfactants (e.g., SDS) and BGEs (e.g. borate) commonly used in MEKC are not compatible with MS detection, significant signal suppression occurs along with the contamination of the ion source. This phenomenon necessitates the application of" MS-friendly" surfactants, of which the most promising seems to be perfluorooctanoic acid (PFOA) [22–24].

We propose for the first time, a CE-MS method utilizing MEKC mechanism for the separation of artemisinin and related compounds. The BGE composition was optimized to achieve the best possible resolution for even complex biological samples, such as various plant extracts and a commercially available artemisinin-containing dietary supplement.

2. Material and methods

2.1. Reagents, samples

All chemicals were of analytical grade. Stock solutions of artemisinin, casticin and deoxy artemisinin obtained from Sigma-Aldrich (St. Louis, MO, USA) were freshly prepared and diluted with ethanol prior to use. Ammonium formate, ammonium acetate, methanol, ethanol and isopropanol were purchased from VWR (Radnor, PA, USA). Ammonium salt of PFOA was purchased from Sigma-Aldrich and it was first dissolved in a small amount of isopropanol and then was diluted with water (Millipore Synergy UV, 18.2 MΩ). All the solutions were filtered using a membrane filter of 0.45 μm pore size and stored at +4 °C. Running buffers and standard solutions were degassed in an ultrasonic bath for at least 5 min. Prior to first use the fused silica capillary was rinsed with 1 M NaOH for 5 min, 0.1 M NaOH for 10 min and finally with de-ionized water for 10 min.

In this study, *Artemisia annua* leaf samples were collected from three different habitat of Debrecen-Nagyerdő (Hungary) and identified by taxonomic criteria noted by Polyakov and Shishkin [25]. The three collected Artemisia samples are abbreviated as AS1-AS3 in this study. Artemisia samples were subjected to drying in warm flowing air for 3 h (temperature not exceeding 40 °C) within an hour after collection. Residual humidity was removed by overnight lyophilization in a Christ Alpha 1–2 LD apparatus. Samples were stored in sealed vials, in darkness, at room temperature until further processing. The dried samples were homogenized using a mortar and pestle using liquid nitrogen. 25 mg was thoroughly mixed with 1 mL ethanol, maintained at room temperature for 10 min and extracted for 1 h at 75 °C. The mixture was centrifuged at 13,000 rpm for 3 min and the obtained supernatant was stored at −24 °C. The commercial dietary supplement Artemizia® was obtained from Hymato Products Ltd, Hungary.

2.2. Instrumentation

The CE-MS measurements were carried out using a capillary electrophoresis instrument (7100 CE System, Agilent, Waldbronn, Germany) coupled to an electrospray mass spectrometer (maXis II UHR ESI-QTOF MS, Bruker, Karlsruhe, Germany). Hyphenation was performed with a CE-ESI sprayer interface (G1607B, Agilent). Sheath liquid (SL) was transferred with a 1260 Infinity II isocratic pump (Agilent). CE instrument was operated by OpenLAB CDS Chemstation software.

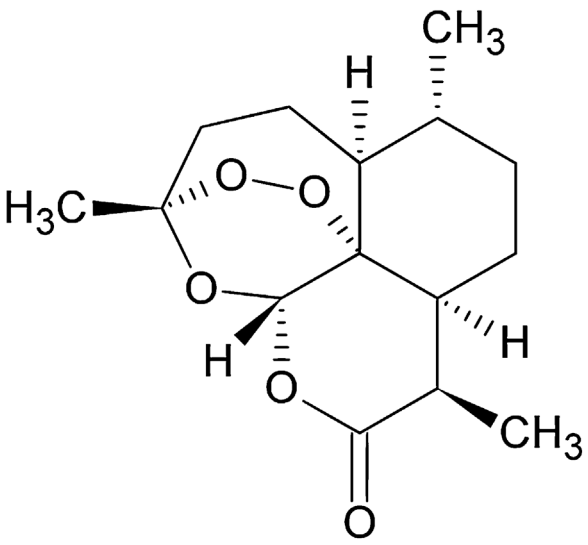

Fig. 1. The structure of artemisinin.

Parameters for the capillary zone electrophoretic separation: capillary: 90 cm ×50 μm fused silica; BGE: 40 mM ammonium formate (pH 9.5); applied voltage: 25 kV; hydrodynamic injection: 50 mbar x 6 s; SL: 50 % (v/v) isopropanol:water containing 0.1 % formic acid; SL flow rate: 7.5 μL/min. The optimized BGE for the MEKC separation of artemisinin contained 40 mM PFOA/NH3 of pH = 9.5 with 2% IPA. The electric current during the CE separation was recorded by ChemStation v. B.04.02 software (Agilent).

The following parameters were applied to the electrospray ion source (positive ionization mode): capillary voltage: 3.5 kV; end plate offset: 500 V; nebulizer pressure: 0.6 bar (during the injection it was switched off); dry gas temperature: 250 °C and dry gas flow rate: 4.0 L/min. The MS method was tuned according to the 50–600 *m/z* mass range. Applied spectra rate was 3 Hz. Na-formate calibrant was injected after each separation, which enabled internal calibration. Electropherograms were background corrected and at times extracted at the masses of the examined analytes. Peaks on the extracted ion electropherograms were integrated manually. Electropherograms and mass spectra were recorded by otofControl version 4.1 (build: 3.5, Bruker) and processed by Compass DataAnalysis version 4.4 (build: 200.55.2969).

3. Results and discussion

3.1. Mass spectrometric detection of artemisinin

Since artemisinin is thermolabile and lacks any chromophoric or fluorophoric groups, its sensitivity for spectrophotometric detection is very poor [21]. This is why conversion or derivatization of artemisinin is preferred before the analysis [20]. Mass spectrometry provides the facility of direct detection of artemisin and its analogs. MS can additionally offer the possibility of identification of these components through exact mass and/or fragmentation pattern analysis (Fig. 1).

Since the artemisinin content of plants can be low and other constituents can interfere with the measurement of artemisinin, first ESI-MS detection was studied. In the case of ESI-MS, the artemisinin molecule most frequently "flies" with proton, sodium or potassium depending on the matrix of the sample. When an ethanolic plant extract was measured with ESI-MS, artemisinin could be detected in positive ion mode, the most abundant form being [M + Na]+

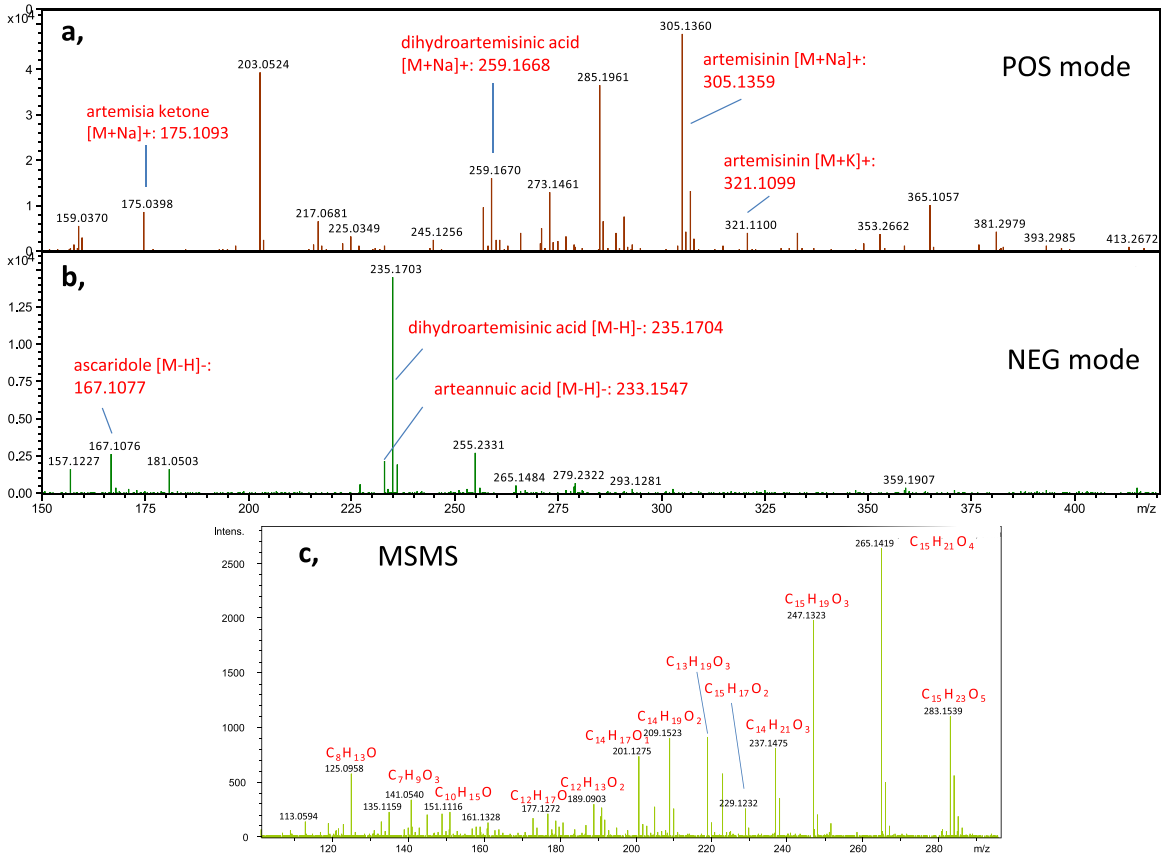

Fig. 2. Mass spectra of plant extract (AS1) using positive (**a,**) and negative ion mode (**b,**). The calculated mass and the detected form of the components were marked in red. Product ion mass spectrum (positive ion mode) of the [M+H]+ ion of artemisinin (**c,**). The *m/z* values of the product ions are marked with the suggested chemical formulas.

(Fig. 2.). The [M + K]+ form could also be observed, albeit with considerably lower intensity; however, the [M+H]+ form was practically not detected at all. By contrast, in pure solutions (standard solutions) the protonated molecular ion was present, as well. Several other analogs (e.g. dihydroartemisinic acid, artemisia ketone) could be detected as [M + Na]+ adducts with high intensities. Although the deprotonated molecular ion ([M−H]-) of artemisinin did not appear, some other analogs (e.g. ascaridole, arteannuic acid, dihydroartemisinic acid) gave relatively intense signals in negative ionization mode. During the MEKC-MS analysis the separated components reach the MS ion source without the matrix of the sample but conjointly with the running electrolyte and the sheath liquid. The interferences from BGE and SL constituents (perfluorooctanoic acid, ammonium acetate, isopropanol) were negligible and due to the minimal sodium content, the [M+H]+ species of the components were in abundance.

In addition to the measured exact mass (error<1 ppm) the compounds could be identified by their fragmentation patterns, as well. In Fig. 2.c the MSMS spectrum of the [M+H]+ ion of artemisinin (precursor ion) subjected to CID is shown. The obtained fragmentation pattern corresponds well with the hypothetical and measured patterns (formation of ions at *m/z* 265.142, 247.132, 229.123, 209.152, 201.127, 125.096 as the highest intensity fragments) described by others [13,26]. The larger mass fragments were mainly formed by dehydrations of the precursor ion. Similarly to the findings of Nieuwerburgh et al. [25], the [M+H]+ ions were much more easily fragmented (7.5−10 eV collision energies) and yielded larger *m/z* value fragments (which are more specific to the precursor) compared to the fragmentation of the [M + Na]+ adduct (Fig. ESM-1).

3.2. Analysis of artemisinin by capillary electrophoresis – mass spectrometry

Artemisinin is poorly soluble in hydrophobic solvents or water and only slightly soluble in strong polar solvents (e.g. methanol or acetonitrile). Medium polarity solvents such as chloroform or ethanol have good dissolving ability for artemisinin [8]. (At 278 K the mole fraction solubilities of artemisinin are $4.5 \cdot 10^4$ and $1.1 \cdot 10^3$ in 14 % and 100 % ethanol, respectively. [27])

The dissolved artemisinin cannot be ionized in solution, therefore, its effective mobility is zero. However, other analogs of artemisinin and most of the flavonoids which can be present in the plant extracts can be ionized in basic conditions. Hence, the most favourable condition for the separation of these plant extract components is the use of a high pH, MS compatible BGE, such as 40 mM NH_4+/NH_3 (pH = 9.5). In this zone electrophoretic measurement, a moderate separation of the components could be observed, but artemisinin (being electrically neutral) comigrated with other neutral compounds (Fig. 3.a). The addition of 20 % ethanol to the BGE reduced the EOF and so shifted the migration times of the components, but since the peaks were slightly broadened, overall there was no considerable improvement in the resolution of the ionized components (Fig. 3.b and Fig. ESM-2).

The separation of artemisinin from other neutral components could be achieved by MEKC, which utilizes additional chromatographic mechanism besides the electrodriven separation (Fig. 3.c and Fig. ESM-4). While 16 well detectable peaks could be observed with CZE-MS, 19 peaks were separated with MEKC-MS. The extra peaks for MEKC are the components which comigrated with artemisinin in CZE. Because even the charged components have

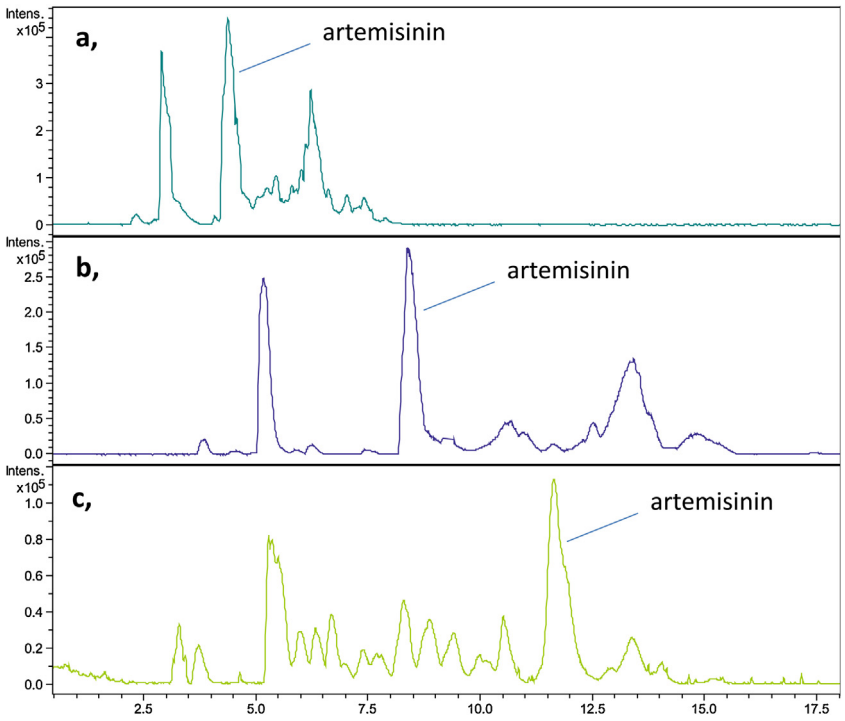

Fig. 3. Base peak electropherograms of plant extract (AS1) obtained by capillary zone electrophoresis using 40 mM NH_4+/NH_3 BGE of pH = 9.5 (**a,**) and 40 mM NH_4+/NH_3 BGE of pH = 9.5 with 20 % ethanol (**b,**) and by micellar electrokinetic capillary chromatography using 40 mM PFOA/NH_3 of pH = 9.5 with 2% IPA (**c,**). Conditions: sheath liquid was 0.1 % formic acid in water:IPA = 1:1; applied separation voltage: +25 kV; hydrodynamic injection: 50 mbar x 6 s; sheath liquid flow rate: 7.5 μL/min, positive MS ionization mode, ESI capillary voltage: 3.5 kV; end plate offset: 500 V; nebulizer pressure: 0.6 bar (during the injection it was switched off); dry gas temperature: 250 °C and dry gas flow rate: 4.0 L/min; mass range: 50-600 *m/z*; spectra rate: 3 Hz.

some hydrophobic moiety, the migration order of the components and the peak patterns of the electropherograms obtained for the same plant extract look quite different for CZE and MEKC.

Ammonium salt of perfluorooctanoic acid was the volatile surfactant of our choice for the MEKC analysis, the use of which was compatible with the ESI-MS system, having hardly any effect on the detection signal in the concentration applied (40 mM), as was stated by others [22]. The obtained peaks are relatively symmetrical and no considerable tailing was observed, since the negatively charged components in the basic electrolyte and the neutral components incorporated in the negatively charged micelles are repelled by the negatively charged capillary surface (a typical phenomenon in MEKC). The application of statically (linearly polymerized acrylamide, (LPA)) or dynamically (polybrene) coated capillaries did not improve the separation efficiency.

Because the pH of the BGE was 9.5, positive ion mode detection demanded the use of acidic SL (0.1 % formic acid content). In order to ensure the complete evaporation of the relatively volatile surfactant (boiling point of PFOA is 190 °C), the drying temperature during ESI was set to 250 °C. It is important to note that despite its thermolability [21], artemisinin did not suffer degradation under such high temperatures in the ESI chamber during the ionization process (Fig. ESM-9).

Following a 10-fold dilution of the plant extract, the peak of artemisinin was split to three overlapping peaks (Fig. 5). The extracted ion electropherogram acquired at the exact mass of artemisinin proved that these peaks should be those of diastereomers. These peaks cannot be originated from optical enantiomers since no chiral selector was incorporated in the electrolyte system; however, the ability of MEKC to separate diastereomers is well-known [28,29]. The MEKC electropherogram of the pure artemisinin standard showed the same 3-peak pattern, and when the diluted plant extract was spiked with the artemisinin standard, all three peaks were increased to a similar degree. It is interesting that the peak patterns of artemisinin were largely different comparing the undiluted and 10-fold diluted plant extracts. The high concentration of artemisinin or the high content of matrix materials of the sample resulted in the appearance of artemisinin as one, two or three peaks, probably, because a large amount of surfactant is consumed for surrounding the hydrophobic components and free micelles are lacking (PFOA has a greater critical micelle concentration but lower solubility than SDS). Artemisinin has several chiral centers, therefore, the existence of diastereomers is very likely (especially in natural products). The pattern of the three overlapped peaks with increasing intensities is probably caused by 4 (2 pairs) diastereomers, of which two were not resolved. Besides artemisinin, (partial) separations of the diastereomers of other components (e.g. artemisinin B, deoxyartemisinin or dihydroartemisinic acid) could be observed, as well.

3.3. Analytical performance and analysis of plant extract

The analytical performance of the developed MEKC-MS system was evaluated for the proposed separation and detection conditions of artemisinin. The calibration diagram showed good linearity (R^2 values were 0.99) in the concentration range of 0.6 μg/mL - 60 μg/mL (Fig. ESM-5). The precision of the analysis was studied by consecutive measurements of the artemisinin standard solution, the RSD% values were 2.6 % for migration times and 4.8 % for peak areas (N = 6) at a concentration of 0.025 mg/mL. The same precision study was performed for plant extract AS1, which contained a large amount of organic compound matrix: the RSD% values were 3.1 % for migration times and 5.6 % for peak areas. In the case of the 10-fold, and 50-fold diluted plant extract samples the RSD% values were quite similar (2.7 % and 3.0 % for migration times and 5.6 % and 5.1 % for peak areas, respectively). The difficulty to precisely integrate the overlapped and tailed peaks of artemisinin or the gradual change of the BGE (PFOA/NH4+ system has low buffer capacity at pH

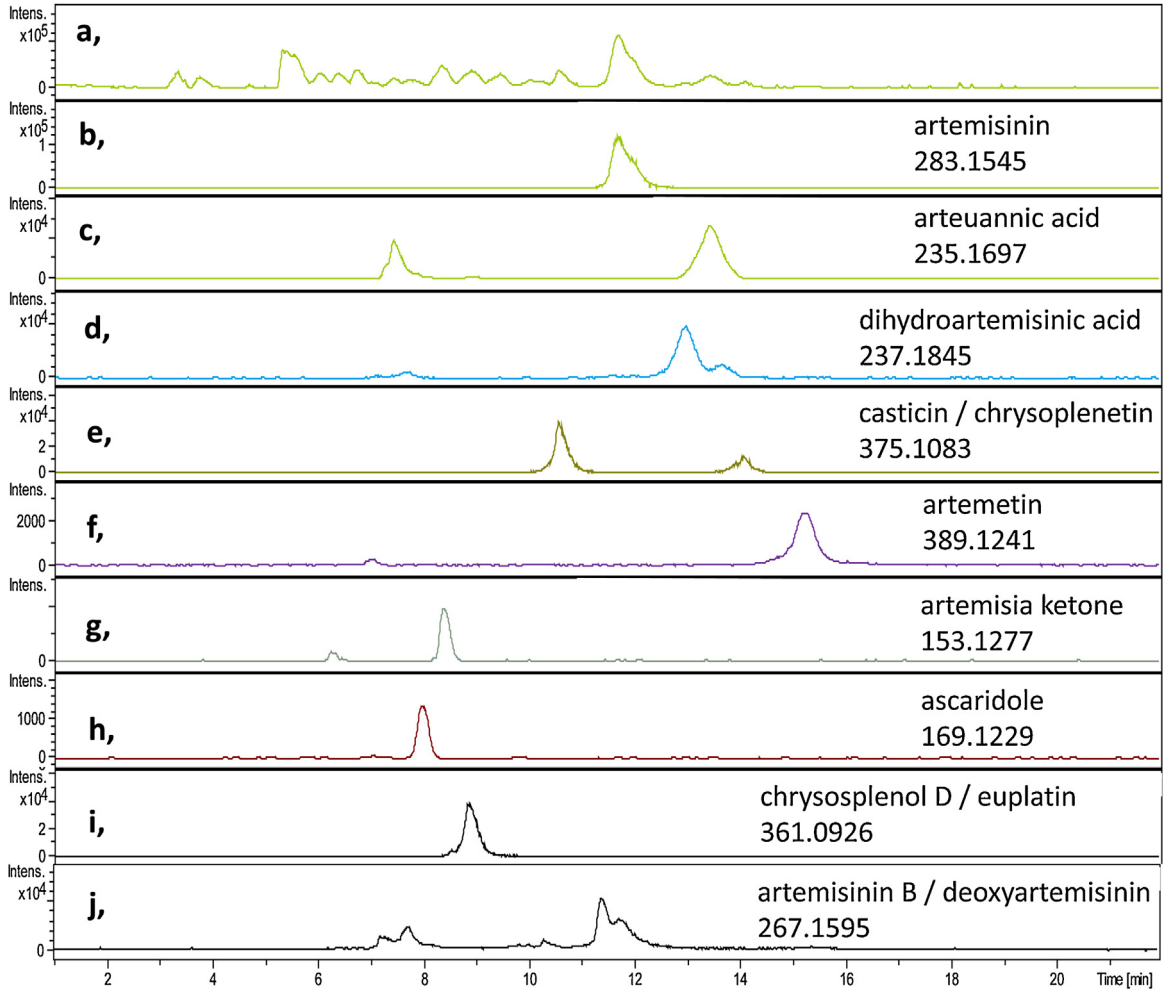

Fig. 4. Base peak (**a,**) and extracted ion (**b-j,**) electropherograms of plant extract (AS1) obtained by MEKC-MS. The MEKC-MS parameters were the same as in Fig. 3.

Table 1
Peak assignments of plant extract (AS1).

	t (min)	m/z	(%)	M calc.	M exp.	Compound	
peak #		[M + H+]+ or [M + Na+]+				name	formula
1	3.763	474.9192	100			unk.	
2	5.308	118.0870	100			unk.	
3	5.989	193.0504	100			unk.	
4	6.367	244.1455	100			unk.	
5	6.702	104.1074	100			unk.	
6	7.442	137.1494	100			unk.	
7	7.945	233.1545	100	232.14578	[M+H]+: 233.1536	epi-deoxyarteannuin B	C15H20O2
	7.735	169.1229	10	168.1145	[M+H]+: 169.1223	ascaridole	C10H16O2
8	8.323	193.0503	100			unk.	
		153.1277	5		[M+H]+: 153.1274	artemisia ketone	C10H16O
9	8.869	361.0926	100	360.08397	[M+H]+: 361.0918	chrysosplenol D	C18H16O8
10	9.466	163.0396	100			unk.	
11	10.037	555.0904	100			unk.	
		499.1237	90			unk.	
12	10.549	375.1082	100	374.0996	[M+H]+: 375.1074	casticin	C19H18O8
	11.666	283.1545	100	282.1462	[M+H]+: 283.1540	artemizinin	C15H22O5
13		209.1540	85			unk.	
	12.909	267.1595	4	266.1518	[M+H]+: 267.1591	deoxyartemisinin	C15H22O4
14	12.960	237.1845	100	236.1776	[M+H]+: 237.1849	dihydroartemisinic acid	C15H24O2
15	13.439	235.1697	100	234.1619	[M+H]+: 235.1692	arteannuic acid	C15H22O2
16	14.203	375.1083	100	374.1001	[M+H]+: 375.1083	chrysoplenetin	C19H18O8
17	15.454	359.1128	100			unk.	
		389.1241	50	388.1152	[M+H]+: 389.1230	artemetin	C20H20O8

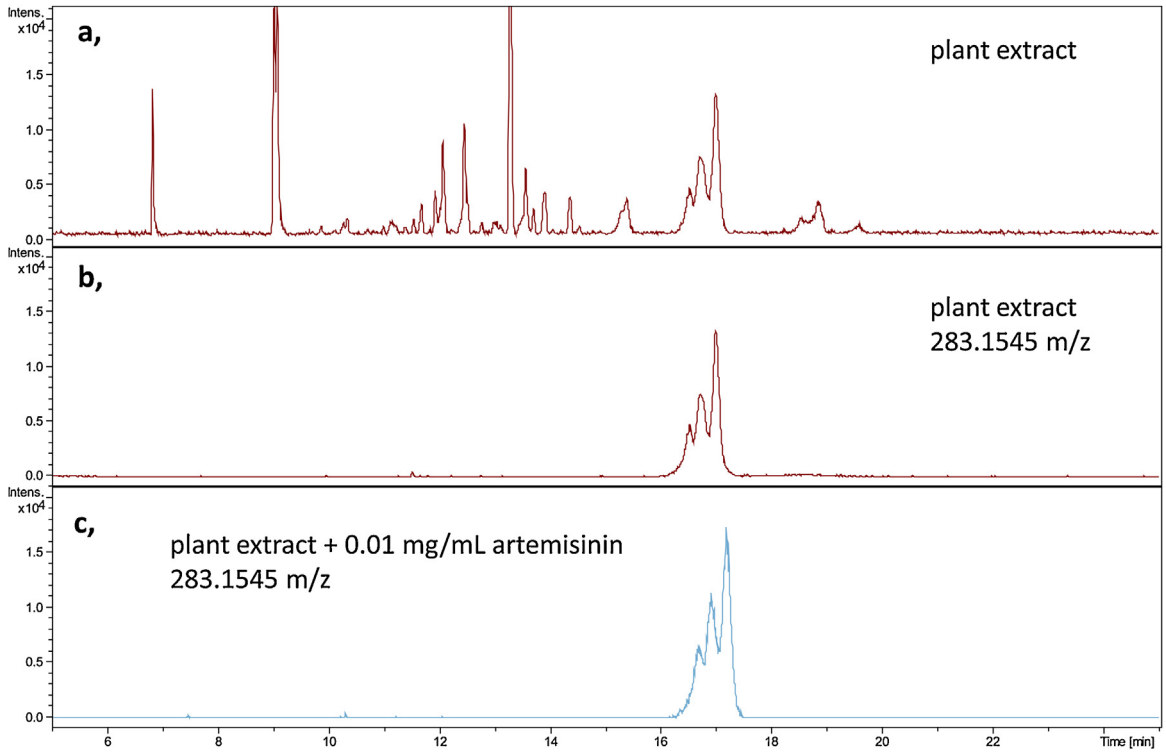

Fig. 5. Base peak (**a,**) and extracted ion (at 283.1545 *m/z*) (**b,**) electropherograms of plant extract (10x diluted AS1) obtained by MEKC-MS. Extracted ion electropherogram (at 283.1545 *m/z*) of plant extract spiked with 0.01 mg/mL artemisinin (**c,**). The MEKC-MS parameters were the same as in Fig. 3.

Table 2
Results of the quantitative analysis of plant extracts (AS1-AS3) and a commercial dietary supplement.

	AS1* mg/mL	AS2*	AS3*	Artemizia ®
artemisinin	0.304	0.214	0.29	13.7
casticin	0.082	0.069	0.089	–
deoxyartemisinin	0.027	0.034	0.025	–

* Contents are shown as the concentrations after sample preparation (multiply by 25 to obtain quantities in mg analyte/g dry plant material).

9.5) could contribute to the relatively poor precision data. The LOD (3S/N) and LOQ (10S/N) values of artemisinin were 0.18 μg/mL and 0.60 μg/mL, respectively. The detection sensitivity could be largely enhanced by increasing the injected sample volume (e.g. extending the injection time from 6 s to 60 s applying 50 mbar pressure), LOD was improved by a factor of 8. (However, such large injection volumes are only allowed in cases where samples contain minimal artemisinin and matrix material.)

In order to test the optimized MEKC-MS method, a plant extract sample (AS1) was analyzed (Fig. 4, Fig. ESM-6). Table 1. provides the qualitative analysis data of artemisinin, its 10 analogs or flavonoids and 12 unknown components. In rare cases, peaks contained more than a single component. The theoretical (calculated) and the experimentally obtained masses typically agreed within 1 ppm accuracy. Similar components with slightly different peak intensities were obtained in additional two plant extract samples (AS2 and AS3) (Fig. ESM-7).

Quantitative analysis was performed for artemisinin and two other constituents (casticin and deoxyartemisinin) in plant extract samples and a commercial therapeutic product (Table 2.). The latter contained only artemisinin from the studied components. In the analyses of the natural extracts, artemisinin was the main (macro) component.

4. Conclusions

Artemisinin and some of its analogs (e.g. ascaridole, artemisia ketone, casticin, deoxyartemisinin, arteannuic acid, artemetin, dihydroartemisinic acid) were determined by MEKC-MS, proving that this still relatively rarely used method is well applicable for the separation and sensitive determination of neutral components lacking chromophore/fluorescent characteristics. The separation of artemisinin from other neutral components was achieved (extra peaks for MEKC are the components which comigrated with artemisinin in CZE), which can be attributed to the nature of the MEKC mode – the joint contribution of chromatographic and electrodriven separation mechanisms (Fig. ESM-8). The application of the micellar technique improved the separation of several charged components, as well. In this work, the main aspects of proper BGE selection for the MEKC-MS determination of artemisinin were demonstrated. The plant extracts could be directly injected into the separation capillary and in addition to artemisinin, a large number of other organic components could be separated and determined. The MEKC-MS revealed the existence of diastereomers of several compounds in the plant extracts.

Author statement
Cynthia Nagy: Methodology, Investigation, Writing
Anna Pesti: Investigation
Melinda Andrasi: Investigation, Methodology
Gabor Vasas: Conceptualization
Attila Gaspar: Conceptualization, Methodology, Writing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114131.

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