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Short communication

Comparison of capillary electrophoresis and high performance liquid chromatography for determination of flavonoids in *Achillea millefolium*

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Abstract

Flavonoids represent an important bioactive component in *Achillea millefolium*. The comparison of the most commonly used analytical methods for the identification and quantification of flavonoids, capillary electrophoresis (CE) and high performance liquid chromatography (HPLC), is presented. The methods were optimized and validated. Using a 20 mM borate buffer with 30% (v/v) of methanol (pH 9.3) in the CE analysis and a gradient elution with water–acetonitrile mobile phase in the HPLC analysis, sufficient separation of the analytes was achieved. A relatively high injection volume in the CE analysis (30 mbar × 30 s) enabled low limit of detection (LOD) (0.3–0.7 mg/L). Repeatability of both methods was acceptable (relative standard deviation of peak area were <6%). Additionally, the amount of flavonoids in a real sample of the dried herbal drug was determined.
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Keywords: *Achillea millefolium*; Capillary electrophoresis; Flavonoids; High performance liquid chromatography

1. Introduction

Achillea millefolium is a well-known medicinal plant, widely used in folk medicine for centuries. Its traditional use includes the symptomatic treatment of bleeding (e.g., caused by hemorrhoids), wounds, gastrointestinal and skin disorders, and hyperhidrosis [1,2]. The German Commission E rates *A. millefolium* positively to treat the lack of appetite, dyspepsia, and cramp-type abdominal pains (internal application) as well as pelvic congestion pains in women (topical application as sitz bath) [3].

The pharmacological activity is attributed to sesquiterpene lactones, azulene, the main constituent of essential oil, and flavonoids. Although the European Pharmacopoeia regulates only the essential oil and proazulene content [4], the presence of flavonoids is of great importance as these substances are known to have a strong spasmolytic [5,6], choleretic [7], antioxidative [8,9], and antimicrobial [9] action. The major flavonoids in *A. millefolium* that were included in our study belong to the class of flavones and flavonoles, and their glycosides: apigenin (API), apigenin-7-*O*-glucoside (A7G), luteolin (LUT), luteolin-7-*O*-glucoside (L7G), and rutin (RUT).

Chromatographic methods such as high performance liquid chromatography (HPLC) [7,10,11] and thin layer chromatography [10,12,13] have been widely used for flavonoid identification and quantification in the genus *Achillea*, and in recent years, capillary electrophoresis (CE) has been recognized as an important alternative or complementary tool [10,12].

The aim of our study was to optimize the CE and HPLC analytical methods for the evaluation of five major flavonoids in *A. millefolium* methanol extract. The results were statistically analyzed using validation parameters (linearity, limits of detection and quantification, accuracy, precision, and robustness) and the methods were applied to determine the amount of flavonoids in a real sample.

2. Experimental

2.1. Materials and reagents

The dried flowering tops of *A. millefolium* L. corresponding to European Pharmacopoeia were obtained from the local pharmacy (Papaja, Kisovec, Slovenia). API, A7G, LUT, L7G, and RUT were obtained from Roth (Karlsruhe, Germany). Sodium

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hydroxide and electrophoresis buffers were from Agilent Technologies (Waldbronn, Germany). Methanol (Merck, Darmstadt, Germany) and acetonitrile (Riedel-de Haën, Seelze, Germany) were of chromatographic grade. All other reagents were of analytical grade.

2.2. Sample preparation

0.5 g of the powdered drug was accurately weighed, extracted with 5 mL of methanol in an ultrasonic bath for 10 min at room temperature, and centrifuged at 11,000 rpm for 10 min. After decantation of the supernatant, the extraction was repeated twice with the same extraction protocol. The supernatants were cumulatively collected and the volume was brought to 15 mL. For a CE analysis, the supernatant was mixed with distilled water (8:2, v/v) and centrifuged at 10,000 rpm for 10 min to avoid precipitation in a capillary due to the weak solubility in an aqueous medium. Stock solutions of flavonoid standards (API, A7G, LUT, L7G, and RUT) were prepared by weighing 10 mg of each flavonoid and dissolving it in 50 mL of methanol. The solutions were then further diluted with methanol to obtain the concentration ranges required (0.5–100 mg/L for each) and stored in a refrigerator. The standard solutions of flavonoids were prepared by mixing the stock solutions of each flavonoid.

2.3. CE

The electrophoretic analyses were performed on a HP^{3D} Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany) controlled by a 3D-CE ChemStation software, Version 10.02. All separations were carried out using an uncoated fused silica capillary 56 cm (50 cm to the detector) × 50 μm i.d. with bubble cell (150 μm) (Agilent Technologies, Waldbronn, Germany). The optimal running conditions: borate buffer (pH 9.3; 20 mM) with 30% (v/v) of methanol, injection 30 mbar × 30 s, voltage 30 kV, temperature 30 °C, detection wavelength 335 nm, analysis time 20 min.

At the beginning of each working day, the capillary was conditioned by flushing with NaOH (1 M) for 3 min, NaOH (0.1 M) for 3 min, and running buffer for 5 min. Between the runs, the following washing steps were performed: 3 min with distilled water, 2 min with NaOH (0.1 M), 2 min with distilled water, and 5 min with the running buffer.

2.4. HPLC

HPLC analyses were performed using a Knauer HPLC system: a Well Chrom K-2500 detector, a Well Chrom K-501 pump, and a Knauer degasser (Knauer Wissenschaftliche Gerätebau, Berlin, Germany), equipped with an EuroChrom® 2000 Basic Edition software, Version 2.05. A C_{18} Kromasil 100 column 4.6 mm × 250 mm, 5 μm (BIA Separations, Ljubljana, Slovenia) was used and detection was performed at 370 nm. The optimal operating conditions: injection volume 12.5 μL, flow rate 0.8 mL/min, mobile phase A distilled water–acetonitrile (81:19, v/v) with 0.1% trifluoroacetic acid (TFA), mobile phase B 100% acetonitrile with 0.1% TFA, elution gradient 0–15 min 100–85% A, 15–20 min 85–75% A, 20–25 min 75–50% A, 25–26 min 50–0% A, 26–40 min 0% A, 40–41 min 0–100% A, 41–50 min 100% A.

3. Results and discussion

3.1. Method optimization

3.1.1. CE

To improve peak resolution, running conditions were optimized varying the running buffer composition and the CE apparatus parameters. Borate (pH 9.3) and phosphate (pH 7.0) buffers were tested at 20 and 50 mM concentration. With the borate buffer, better separation was achieved and the 20 mM concentration was selected for the further studies. Then, the method was additionally optimized to increase resolution of analytes in a drug extract, which is more difficult to achieve due to the complex effects of a real matrix. We examined the influence of different organic modifiers which were reported to improve the resolution of analytes [14–16]. Acetonitrile, ethanol, methanol, propanol, isopropanol, and mixtures of acetonitrile/methanol and ethanol/methanol were tested in concentrations from 10 to 30% (v/v). With the 30% methanol, the highest resolution was achieved. This effect could be attributed to the enlargement of the migration time window and increased solvation of the analytes. In the previous research [12], 20% of methanol in the running buffer was used. In our method, 20% of methanol did not produce sufficient separation, which can be due to approximately 30-fold larger injection volume used. The addition of sodium dodecyl sulfate in concentrations from 1 to 100 mM markedly decreased the resolution, even in the lowest concentrations. Additionally, running voltage (15–30 kV), capillary temperature (20–35 °C), injection time (5–40 s), and injection pressure (20–30 mbar) were tested. The electropherograms obtained using optimal conditions are shown in Fig. 1.

3.1.2. HPLC

For the HPLC method optimization, the following operating conditions were varied: mobile phase, elution gradient, and column parameters (injection volume and flow rate). The most commonly used mobile phases for the separation of flavonoids consist of an organic acetonitrile and/or methanol phase, and a water phase with TFA [17,18]. In our experiments, the methanol (as used in [10]) and methanol–acetonitrile mobile phases markedly decreased the resolution in comparison to the acetonitrile mobile phase (also used in [7] and [11]). The separation under optimal conditions resulted in chromatograms shown in Fig. 2.

3.1.3. Extraction

The amount of API, A7G, LUT, L7G, and RUT was determined after each of the five successive extraction steps. As shown in Fig. 3, the major part (approximately 90% of the total amount) of flavonoids was extracted in the first three extraction steps. Therefore, the combined extracts of the three successive extractions were used for the determination of flavonoids in *A. millefolium* real drug sample.

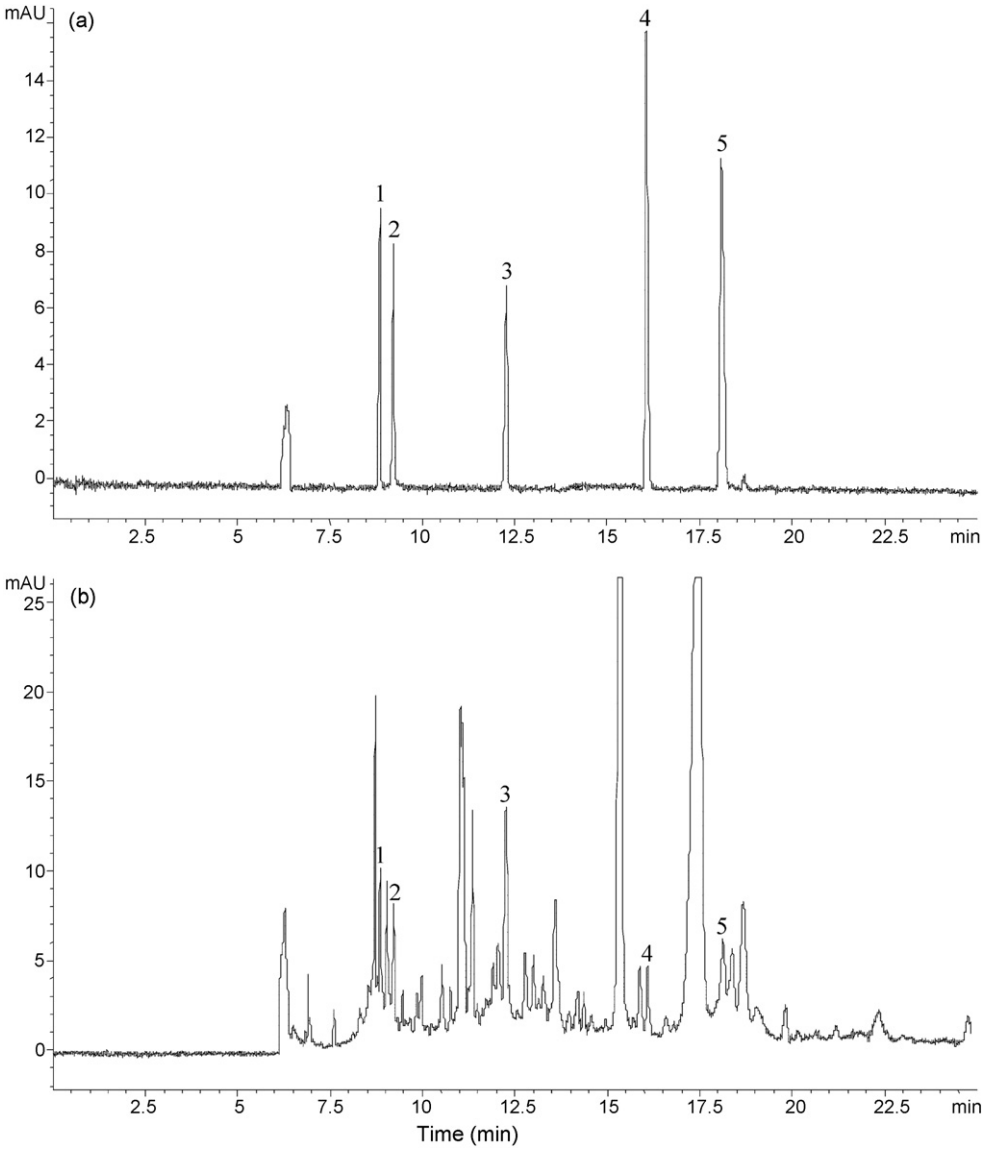

Fig. 1. Electropherograms of a standard solution of flavonoids in methanol at a concentration of 10 μg/mL (a) and of the combined supernatants of three successive methanol extractions of *Achillea millefolium* drug (b) obtained using optimal conditions (borate buffer (pH 9.3; 20 mM) with 30% (v/v) of methanol, injection 30 mbar × 30 s, voltage 30 kV, temperature 30 °C). The peaks identified: A7G (1), L7G (2), RUT (3), API (4), and LUT (5).

3.2. Validation of the methods

The characteristics and the procedures used for validation were those described in USP 30 [19] and in the International Conference of Harmonization (ICH) Guidelines (Q2A, Q2B) [20,21]. Also some other literature data were used [22–24].

The peaks were identified by comparison of retention time and UV spectra with standards and spiking of extracts with standards.

We studied linearity in a range of 0.5–100 mg/L (0.5, 1.0, 3.0, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0 mg/L) for both methods. In addition, the results for the limit of detection (LOD—S/N ratio 3:1) and the limit of quantification (LOQ—S/N ratio 10:1) of each compound were determined and are shown in Table 1. LOD in our method was approximately 10-fold lower compared to LOD in the previous research [12], which is due to larger injection volume used in our method.

Accuracy of the method was determined by analyzing standard solutions of known concentrations. The mean recoveries for all compounds were in the range of 99.1–101.0 and 99.9–100.7% for HPLC and CE, respectively ($n = 6$ for each of presented concentration), proving a good accuracy of both methods (Table 1).

Repeatability test was performed by determination of the intraday variation in peak's areas and migration/retention times using standard solutions. The R.S.D. values for peak area were below 5.1 and 4.5% for HPLC and CE, respectively ($n = 6$), and for migration/retention times $\leq$0.9% for both methods, which indicates that the repeatability of the methods is acceptable (Table 2) and similar to repeatability of the previously published method [12]. Intermediate precision was evaluated

Table 1
Statistical parameters of the calibration curve (linear regression) for each compound, with LOD and LOQ values, and determination of accuracy in samples of known concentration

	API		A7G		LUT		L7G		RUT	
	CE	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE	HPLC
Slope	6.029	0.128	2.016	0.108	5.892	0.192	2.032	0.183	1.899	0.133
Intercept	−1.844	−0.015	−0.874	−0.008	−2.525	−0.019	−1.271	−0.126	−0.875	0.003
R^2	0.9988	0.9996	0.9990	0.9996	0.9989	0.9993	0.9989	0.9987	0.9988	0.9990
LOD (mg/L)	0.5	0.4	0.3	0.8	0.6	0.2	0.4	0.6	0.7	0.9
LOQ (mg/L)	1.6	1.3	0.9	2.7	2.0	0.8	1.5	1.8	2.3	3.0
Linear range (mg/L)	2–100	1–100	1–100	3–100	2–100	1–100	2–100	2–100	2–100	3–100
Recovery ± S.D. (%)	99.95 ± 2.35	100.24 ± 1.76	100.26 ± 2.48	99.82 ± 2.07	99.93 ± 2.22	99.61 ± 2.58	100.66 ± 3.90	101.01 ± 4.58	100.56 ± 3.79	99.16 ± 3.97

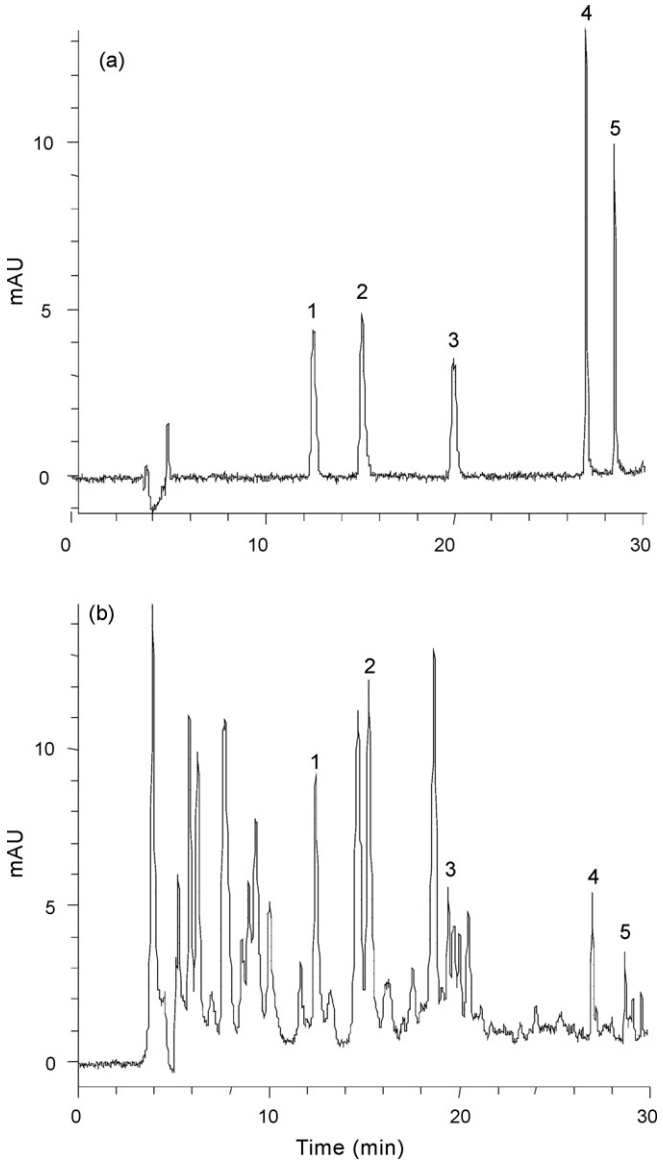

Fig. 2. Chromatograms of a standard solution of flavonoids in methanol at a concentration of 10 μg/mL (a) and of the combined supernatants of three successive methanol extractions of *Achillea millefolium* drug (b) obtained using optimal conditions (injection volume 12.5 μL, flow rate 0.8 mL/min, mobile phase A distilled water–acetonitrile (81:19, v/v) with 0.1% TFA, mobile phase B 100% acetonitrile with 0.1% of TFA, elution gradient 0–15 min 100–85% A, 15–20 min 85–75% A, 20–25 min 75–50% A, 25–26 min 50–0% A, 26–40 min 0% A, 40–41 min 0–100% A, 41–50 min 100% A). The peaks identified: RUT (1), L7G (2), A7G (3), LUT (4), and API (5).

over 3 days (the inter-day repeatability; $n = 6$) using standard solutions (concentrations 2.5–25.0 mg/L). These solutions were injected daily under the same conditions and the results were used for the repeatability study. The solutions were stored at room temperature (25 ± 2 °C) in diffuse daylight, decreasing recovery values approximately from 101.0 to 97.3% for all compounds in water/methanol. When stored in a refrigerator in the dark, the recovery ranged from 101.1 to 99.7% over 3 days for all compounds. The R.S.D. values (0.8–1.2 and 0.7–1.0% for migration/retention time and 1.6–3.7 and 2.8–3.7% for peak area

Table 2
Determination of repeatability and intermediate precision (Pa: peak area; t_m: migration time; t_r: retention time)

Sample	Parameter	R.S.D. (%)									
		Repeatability ($n=6$)					Intermediate precision (3 days; $n=6$)				
		API	A7G	LUT	L7G	RUT	API	A7G	LUT	L7G	RUT
Standard solution	CE (Pa)	3.08	3.57	3.41	**4.45**	4.29	1.94	2.91	1.56	**3.67**	2.76
	HPLC (Pa)	3.32	4.68	2.89	4.34	**5.11**	3.24	2.83	**3.71**	2.99	3.62
	CE (t_m)	0.81	0.85	0.67	**0.93**	0.75	0.78	0.95	**1.18**	1.05	0.98
	HPLC (t_r)	0.49	0.52	0.62	**0.89**	0.56	0.86	0.69	0.94	**0.99**	0.83
Drug extract	CE (Pa)	4.71	3.16	5.53	**5.77**	4.14	2.17	2.19	4.28	3.17	**5.05**
	HPLC (Pa)	4.46	4.30	2.40	5.39	**5.83**	2.10	**5.11**	2.97	4.05	5.21
	CE (t_m)	0.79	0.88	1.02	**1.13**	1.08	0.89	0.72	1.21	**1.32**	1.05
	HPLC (t_r)	0.65	0.61	0.59	0.66	**0.74**	1.02	1.07	**1.11**	0.95	0.98

The bold values represent the maximum RSD values for repeatability and intermediate precision, respectively, which is clearly explained in the text.

for CE and HPLC, respectively) indicate that the intermediate precision is acceptable (Table 2).

Additionally, the precision of the methods was also examined by six replicate injections of the methanol extract intraday and between 3 days. The repeatability and intermediate precision for peak area (Table 2) was acceptable for each compound and did not exceed 5.8 and 5.0% (CE), and 5.8 and 5.1% (HPLC), respectively, as well as for migration/retention time which was below 1.1 and 1.3% (CE), and 0.7 and 1.1% (HPLC), respectively.

The parameters of the optimum CE and HPLC conditions were slightly modified in order to evaluate the robustness [24]. The design applied was the fractional factorial design. The effects of different concentrations of an organic modifier (±0.5%) in the running buffer as well as the effects of buffer pH (±0.06), capillary temperature (±5 °C), running voltage (±1 kV), and detection wavelength (±3 nm) were determined for the CE method. No significant variations in specificity, accuracy, and precision were found over the tested ranges, which indicated a good robustness of the CE method (R.S.D.s were lower then 1.9% for migration time and peak area).

The effects of different concentrations of an organic modifier (±0.5%) in the mobile phase as well as the effects of pH (±0.06), column temperature (±5 °C), flow rate (±0.1 mL/min), and detection wavelength (±3 nm) were determined for the HPLC method. No significant variations in specificity, accuracy, and precision were found over the tested ranges, which indicated good robustness of the HPLC method (R.S.D.s were lower then 1.2% for retention time and peak area).

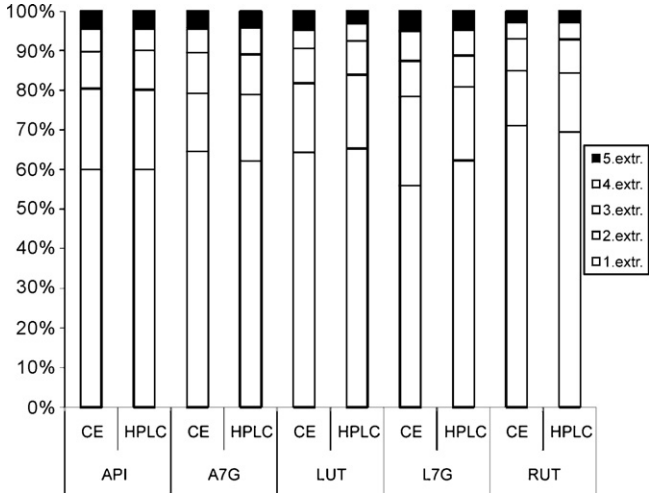

Fig. 3. The amount of flavonoids after each of the five successive extraction steps determined by CE and HPLC.

Table 3
Application results ($n=6$)

	Flavonoid/dry drug (mg/g)				
	API	A7G	LUT	L7G	RUT
CE	0.09	0.17	0.13	0.32	0.54
HPLC	0.10	0.15	0.13	0.47	0.48

3.3. Application

The presented CE and HPLC methods were tested to determine the five flavonoids in a real sample of the dried herbal drug. The results for all five flavonoids in a real sample are shown for both CE and HPLC methods in Table 3. The presented results are comparable with the reported values [12].

4. Conclusions

The advantage of the proposed CE method over the HPLC method for the analysis of flavonoids in *A. millefolium* is its lower running costs and better environmental acceptability. In the developed and proposed methods, 20 analyses with CE require 2 mL of the borate buffer containing 30% (v/v) of methanol, while 20 analyses with HPLC require approximately 600 mL of the mobile phase with acetonitrile. A disadvantage of a CE method is usually a lower sensitivity in contrast to a HPLC method, but in our study under optimal conditions for the analysis of flavonoids from *A. millefolium*, the difference in sensitivity was not significant between the proposed methods. The HPLC method showed slightly better robustness over the CE method under developed and optimal conditions.

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