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Validation of a Capillary Electrophoresis Method for the Quantitative Determination of Free and Total Apigenin in Extracts of *Chamomilla recutita*

Fabiana N. Fonseca and Marina F. M. Tavares*

Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes 748, São Paulo-SP, 05508-900, Brazil

A capillary electrophoretic method for the quantification of free and total apigenin in methanolic, ethanolic and glycolic extracts of *Chamomilla recutita* L. Rauschert (Asteraceae) is described. The method was validated for measurement of apigenin in the range 5.00–300 μg/mL (r^2 = 0.993) and showed coefficients of intra-day (replicability) and inter-day (repeatability) variability of better than 2%. The limits of detection and quantification were 3.80 and 11.5 μg/mL, respectively, and the average recovery was 102.0 ± 0.8% at three concentration levels of apigenin. Free and total apigenin contents in the extracts were, respectively, determined as 106 and 903 μg/g (methanolic extract), 77 and 817 μg/g (ethanolic extract) and 11.0 and 247 μg/g (glycolic extract).

Keywords: Capillary electrophoresis; method validation; apigenin; herbal extracts; *Chamomilla recutita*.

INTRODUCTION

German chamomile is a herb, native to south-east Europe and west Asia, which has spread throughout Europe, North America and Australia (Evans, 1996). *Chamomilla recutita* L. Rauschert (family Asteraceae) is the legitimate name for the species (Rauschert, 1990; World Health Organisation, 1999), although synonyms such as *Matricaria recutita* L. and *Matricaria chamomilla* L. are often found in formularies and references.

Chamomile flowers contain an essential oil (0.4–1.5%), which is rich in chamazulene, α-bisabolol and related sesquiterpenes (World Health Organisation, 1999), as well as a phenolic fraction in which apigenin and related flavonoid glycosides form the main components, constituting up to 8% (dry weight) of the drug (Kunde and Isaac, 1979). Apigenin does not appear to accumulate in chamomile flowers in the free form (Schreiber *et al.*, 1990), and there is evidence to suggest that the aglycone is actually a product of post-harvest degradation (Maier *et al.*, 1991). Besides apigenin and its derivatives, chamomile flowers also contain luteolin, quercetin, patuletin (and their glycosides), herniarin and umbelliferone (Redaelli *et al.*, 1981a) as well as several phenylpropanoid derivatives (Dondi *et al.*, 1987; Mulinacci *et al.*, 2000).

Extracts of chamomile flower heads are used in both pharmaceutical and cosmetic industries (Vernin *et al.*, 1992) for their established anti-spasmodic, anti-microbial (*British Herbal Pharmacopoeia*, 1989) and anti-inflammatory properties (Della Loggia *et al.*, 1986). The spasmolytic activity is mainly attributed to the presence of apigenin, apigenin-7-*O*-glycoside and its acetyl derivatives (Zekovic *et al.*, 1994), and these flavones are also known to play an important role in the overall anti-inflammatory activity of chamomile extracts (Della Loggia *et al.*, 1986). In general, the pharmacological properties of apigenin are thought to be superior to those of its glycosides (Tuckermann *et al.*, 1980); however, since mammals can hydrolyse the glycosidic bonds of orally administered flavonoid glycosides (Griffiths and Smith, 1972a, b), it appears unimportant whether chamomile extracts are rich in free apigenin or a bound form (Schreiber *et al.*, 1990). For this reason, several authors have proposed the determination of the total amount of apigenin in chamomile flowers instead of analysing each of the apigenin glycosides separately (Briançon and Rotger, 1983; Perez *et al.*, 1994; Zekovic *et al.*, 1994). A simple approach to obtain the aglycone is to submit the extract to a mild acid hydrolysis which releases the glycoside moiety without promoting decomposition of the remaining aglycone skeleton (Vernin *et al.*, 1992). Apigenin and its derivatives have been quantified by gradient-elution HPLC with analysis times varying from 25 min (Redaelli *et al.*, 1981b) up to 50 min (Mulinacci *et al.*, 2000). Although octadecylsilica has been the most commonly used stationary phase, other functionalised silica supports such as octyl and cyano have also been proposed (Mauz *et al.*, 1996; Scalia *et al.*, 1999).

During the last decade or so, capillary electrophoresis (CE) has received considerable attention with respect to the analysis of secondary metabolites: flavonoids, phenylpropanoids, coumarins, phenolic acids and alkaloids have all been characterised using this technique (Pietta *et al.*, 1991; Tomás-Barberán, 1995; Suntornsuk, 2002). However, despite the numerous publications

* Correspondence to: M. F. M. Tavares, Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes 748, São Paulo-SP, 05508-900, Brazil.
Email: mfmtavar@iq.usp.br
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describing the use of CE for the analysis of herbal extracts, very few have emphasised the advantages of the technique for the quality control of such complex matrices. The present paper describes a methodology for the quality control and standardisation of extracts of *C. recutita* based on the analysis of apigenin contents. The method has been validated according to the protocol of the *United States Pharmacopoeia* (2002) and applied to the determination of free and total apigenin in methanolic, ethanolic and glycolic chamomile extracts.

EXPERIMENTAL

Reagents and solutions. Reagents were of analytical grade and solvents were of chromatographic purity. The water used to prepare the solutions was purified and deionised using a Milli-Q system (Millipore, Billerica, MA, USA). The electrolyte employed consisted of a 0.020 M tetraborate solution adjusted to pH 10 with 1 M sodium hydroxide. Electrolyte solutions were prepared fresh daily and filtered through a 0.45 μm membrane filter (Millipore) prior to use.

Instrumentation. All experiments were conducted using an Agilent (Palo Alto, CA, USA) model HP^{3D}CE capillary electrophoresis system equipped with a diode array detector set at 337 nm, a temperature control device maintained at 25°C and an HP ChemStation with data acquisition and treatment software (revision A.06.01) supplied by the manufacturer. The fused-silica capillary (total length 63 cm; effective length 54.5 cm; 75 μm i.d.; 365 μm o.d.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). The electrophoresis system was operated under normal polarity and constant voltage conditions of +25 kV. Samples and standard solutions were injected hydrodynamically (50 mbar for 3 s).

Analytical procedures. At the start of each day, the capillary was conditioned by a pressure flush (930 mbar) of 1 M sodium hydroxide solution (20 min), deionised water (10 min) and electrolyte solution (30 min) followed by an electrokinetic flush of electrolyte (+25 kV for 15 min). Between runs, the capillary was replenished with fresh electrolyte solution with a pressure flush (1 min) followed by an electrokinetic flush (+25 kV; 4 min).

Reference standards. Umbelliferone, caffeic acid, chlorogenic acid, apigenin, luteolin, rutin and quercetin standards were purchased from Sigma (St. Louis, MO, USA); apigenin-7-*O*-glycoside and luteolin-7-*O*-glycoside were obtained from Extrasynthèse (Genay, France) and herniarin was purchased from Fluka (St. Louis, MO, USA). Stock solutions of the standards in dimethylsulphoxide:methanol (1:9) were prepared weekly at concentrations of 1000 μg/mL and stored in the dark in a freezer.

Samples. Seeds of *Chamomilla recutita* L. were obtained from a local farm (Fazenda Demetria, Botucatu, SP, Brazil) and planted in early April 1998 (Seara, Jacupiranga, SP, Brazil). Flowers were harvested from July throughout August of the same year. Professor Lin Chau Ming (Universidade Estadual Paulista, Botucatu, SP, Brazil) confirmed the authenticity of the samples. Flower heads, in the late stage of development, were air-dried (40°C) to constant weight, and a sample (16.0 ***g***) extracted with 400 mL of methanol:water (1:1) for 1 h with sonication. The extraction procedure was repeated in triplicate, the extracts bulked, filtered and evaporated completely under vacuum. In order to isolate the phenolic fraction of the extract, the residue was suspended in 24.00 mL of methanol and an aliquot (10.00 mL) diluted in 20.00 mL of water to obtain a stock extract solution. Samples to be analysed for free apigenin were prepared by accurately weighing 5 ***g*** of the stock extract solution into a 10 mL volumetric flask and completing the volume with methanol:water (1:1).

Ethanolic and glycolic extracts of *C. recutita* were obtained locally from Farma Service Indústria Farmacêutica Ltda (São Paulo, SP, Brazil: batches FS65521098 and FS54481197, respectively), each containing ca. 10% (w/w) of floral heads. For the ethanolic extract, ethanol (70%) in water was the extracting solvent, whilst for the glycolic extract, propylene glycol (92.7%, w/w), glycerine (2%, w/w) and ethanol (5%, w/w) was employed. In both cases, methyl-4-hydroxybenzoate (0.2%, w/w) and propyl-4-hydroxybenzoate (0.1%, w/w) were incorporated as preservatives. Samples to be analysed for free apigenin were prepared by accurately weighing 5 ***g*** of the ethanolic extract or 2.5 ***g*** of the glycolic extract and diluting to 10 mL with methanol:water (1:1) in a volumetric flask.

Hydrolysis. Chamomile extracts were hydrolysed in acidic medium following the procedure of Martini and Seiller (Vernin *et al.*, 1992) with minor modifications. Approximately 1 mL of concentrated hydrochloric acid was added to an aliquot (5.00 mL) of the stock solution of a methanolic extract, or of the diluted solution of an ethanolic or glycolic extract, and the mixture was heated to reflux (ca. 100°C) for 1 h. After this time, the solution was cooled and the phenolic fraction was extracted five times with 6 mL portions of diethyl ether. The fractions were combined, dried over sodium sulphate and the solvent completely evaporated. The residue was dissolved in 1 mL methanol, transferred to a 5 mL volumetric flask, and the volume completed with methanol:water (1:1). An aliquot (1.50 mL for methanolic and ethanolic extracts, 1.00 mL for glycolic extracts) of this solution was again diluted to 5.00 mL, filtered, sonicated and reserved for analysis of the total apigenin content. All hydrolysis procedures were performed in triplicate.

Validation of method. For the construction of calibration curves, appropriate aliquots of the standard stock solution of apigenin (1000 μg/mL) were transferred to separate 10 mL volumetric flasks and the volumes were completed with deionised water to yield final concentrations of 5.00, 10.0, 25.0, 50.0, 75.0, 100, 150, 200, 250 and 300 μg/mL of apigenin. All solutions were sonicated for 10 min, and filtered through a 0.22 μm filter (Millipore) prior to injection. Each solution was analysed in triplicate, and the resulting peak areas were plotted against the respective apigenin concentrations. The accuracy of the method was evaluated by recovery tests in which samples of the hydrolysed methanolic extracts were spiked with standard apigenin solutions at three different concentrations, namely, 25.0, 50.0 and 75.0 μg/mL, according to Table 1.

Table 1. Constitution of samples employed for the recovery tests

Standard apigenin solution[a] (μL)	Methanol (μL)	Hydrolysed methanolic extract (μL)
0	100	900
25.0	75.0	900
50.0	50.0	900
75.0	25.0	900

[a] Standard solution contained 1000 μg/mL of apigenin.

RESULTS AND DISCUSSION

In a previous study (Fonseca *et al*., 2001), the electrolyte composition was optimised for the separation of eleven phenolic compounds using both free solution and micellar electrokinetic chromatography. Both methods proved to be useful in the identification of various phenolic compounds in chamomile extracts. In the present work, a 0.020 M tetraborate solution (pH 10) was selected for the quantitative evaluation of free and total apigenin in chamomile extracts from a number of sources. The proposed CE methodology was fully validated according to the *United States Pharmacopoeia* (2002), including parameters such as specificity, linearity, limit of detection, limit of quantification, accuracy and precision.

Specificity

Specificity is the ability unequivocally to determine the analyte in the presence of other components, typically, impurities, decomposed products, matrix constituents etc. The specificity of the proposed methodology was demonstrated by showing the non-interference by other phenolic compounds, expected to be present in an extract of chamomile, with the measurement of apigenin. A number of pure standards of such phenolic compounds were analysed under the conditions of the proposed methodology, and the on-line UV spectra were recorded for each peak (data not shown). The baseline separation of the 11 standards employed has been presented elsewhere (Fonseca *et al.*, 2001).

Figure 1(A–F) shows electropherograms of methanolic, ethanolic and glycolic extracts of chamomile before and after being submitted to the acidic hydrolysis procedure described. Hydrolysis considerably simplified the electrophoretic profile of the extracts, resulting in a clear separation of the apigenin peak from the other sample constituents. Identification of apigenin in the actual extracts was performed by spiking techniques and by performing peak purity index analysis: all of the peaks presented purity indices superior to 998, confirming the specificity of the method for apigenin.

It may also be observed from Fig. 1(A–F) that, besides apigenin, the levels of caffeic acid and luteolin increased significantly upon hydrolysis, whereas quercetin and umbelliferone increased only modestly (except for quercetin in the methanolic extract, which decreased). Additionally, all hydrolysed extracts contained a unidentified compound eluting in ca. 5.4 min (labelled '#' in the electropherograms), which showed a spectral resemblance to chlorogenic acid. This evidence, along with a

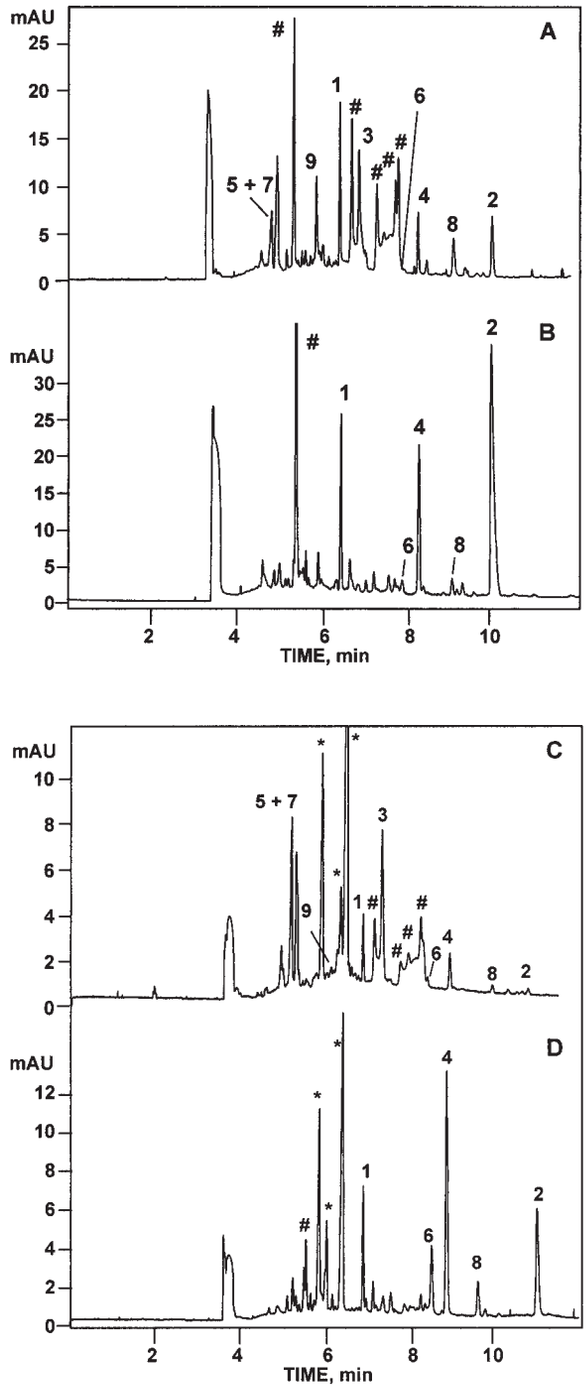

Figure 1. Capillary electropherograms of various extracts of *Chamomilla recutita* L. showing: methanolic extracts (A) before and (B) after hydrolysis; ethanolic extracts (C) before and (D) after hydrolysis; and glycolic extracts (E) before and (F) after hydrolysis. Key to peak identities: **1**, umbelliferone; **2**, caffeic acid; **3**, chlorogenic acid; **4**, apigenin; **5**, apigenin-7-*O*-glycoside; **6**, luteolin; **7**, luteolin-7-*O*-glycoside; **8**, quercetin; **9**, rutin; peaks labelled with '#' are tentatively assigned as chlorogenic acid derivatives; peaks labelled with asterisks are associated with the preservatives present in the extract. (For analytical protocol see the Experimental section.)

recent report (Mulinnacci *et al.*, 2000) of high levels of hydroxycinnamic acid derivatives in chamomile flowers, suggest that the unidentified peak may be the aglycone of a chlorogenic or ferulic acid derivative. However, taking into consideration that the hydrolytic conditions were selected only for apigenin, and may not have been appropriate for other components, any comparison of

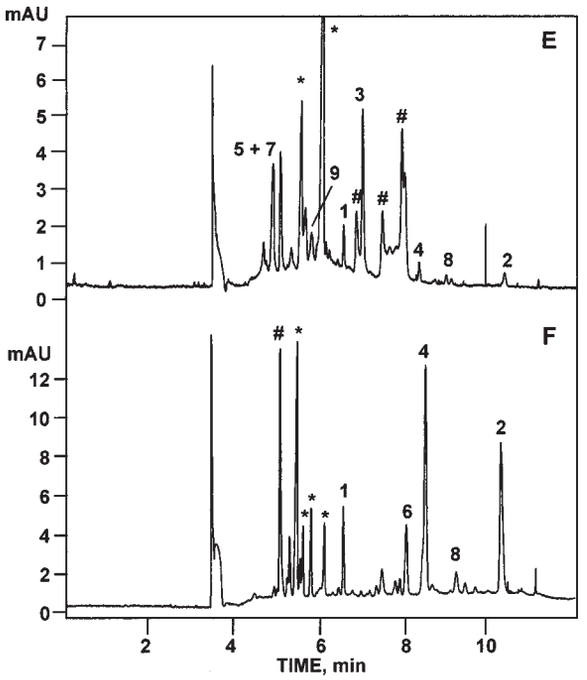

Figure 1. *Continued*

the relative abundance of aglycones in the extract might be in error. Studies to optimise the hydrolytic conditions for the quantitative determination of the main phenolic aglycones in chamomile extracts are currently under investigation in our laboratory.

Linearity, limits of detection and quantification

The interval of linear response of the detector with respect to apigenin covered the concentration range from 5.00 to 300 μg/mL ($y = 1.027\,x + 1.553$; $r^2 = 0.993$), although a calibration curve comprising a smaller concentration interval (5.00–75.0 μg/mL) was used for quantitative purposes. The coefficient of determination and the regression equation were calculated using linear least-square regression analysis as shown by the statistical data in Table 2. An acceptable coefficient of correlation of 0.99 or greater and an intercept close to the origin should be achieved (Swartz and Krull, 1998; *United States Pharmacopoeia*, 2002). The described method showed excellent linearity ($r^2 = 0.9988$) between peak area and apigenin concentration. The limits of detection and quantification were 3.80 and 11.5 μg/mL respectively. The criterion used to determine these limits was based on the ratio of the standard deviation (SD) of the intercept to the calibration curve slope (S), whereby the limit of detection was taken as 3.3 times this ratio and the limit of quantification was 10 times this ratio (Swartz and Krull, 1998).

Table 2. Calibration and validation (linearity and limits of detection and quantification) of the capillary electrophoresis method of determination of apigenin in extracts of chamomile

Parameter	Statistical data
Concentration range[a] (μg/mL)	5.00–75.0
Intercept	1.047
Slope	1.008
Coefficient of determination (r^2)	0.9988
Standard deviation of intercept	1.16
Limit of detection (μg/mL)	3.80
Limit of quantification (μg/mL)	11.5

[a] Relates to pure standard solutions.

Accuracy

In the absence of a certified standard for the determination of apigenin, accuracy was evaluated by recovery tests. Samples of the hydrolysed methanolic extract were spiked with standard apigenin solutions at concentrations of 25.0, 50.0 and 75.0 μg/mL and then analysed by CE. Table 3 shows the accuracy of the method, with recoveries for apigenin varying from 101.3 to 102.8% at the three concentration levels tested.

Precision

The replicability was determined by the injection of ten replicate samples of the hydrolysed methanolic extract sequentially in a single day. Repeatability was estimated by the triplicate injection of the methanolic extract on three consecutive days. Precision was expressed in terms of coefficients of variation (Table 4): the data presented indicate a good agreement among the individual test results. The repeatability of the hydrolysis procedure was also tested by submitting three separate aliquots of the methanolic extract to hydrolysis. Triplicate analysis of apigenin in the hydrolysed samples gave a coefficient of variation better than 2.3%, indicating that the hydrolytic procedure is reliable.

Quantitative determination of free and total apigenin

The validated methodology was applied to the quantification of free and total apigenin in methanolic,

Table 3. Validation of the accuracy of the capillary electrophoresis method of determination of apigenin in extracts of chamomile: recovery tests performed on the hydrolysed methanolic extract

	Sample 1	Sample 2	Sample 3
Apigenin concentration added (μg/mL)	25.0	50.0	75.0
Apigenin concentration found[a] (μg/mL)	25.5	50.6	77.1
Recovery (%)	101.9	101.3	102.8

[a] Mean values ($n = 3$).

Table 4. Validation of the precision of the capillary electrophoresis method of determination of apigenin in extracts of chamomile: tests performed on the hydrolysed methanolic extract

	Intra-day variability (replicability) (n = 10)	Inter-day variability (repeatability) (n = 9; 3 days)
Average apigenin concentration (μg/mL)	57.14	57.06
Standard deviation	0.75	0.97
Coefficient of variation (%)	1.3	1.7
Confidence interval (95% confidence limits) (μg/mL)	55.6–58.6	55.2–59.0

Table 5. Quantitative determination of free and total apigenin in the methanolic, ethanolic and glycolic extracts of *Chamomilla recutita* L.

Sample	Free apigenin (μg/g)	Total apigenin (μg/g)
Methanolic extract[a]	106 ± 3	903 ± 24
Ethanolic extract[b]	11.0 ± 0.5	247 ± 17
Glycolic extract[b]	77 ± 6	817 ± 8

[a] Amount of apigenin related to the dried flowers used to prepare the extract.
[b] Amount of apigenin related to the crude extract (density of ethanolic extract was 1.00038 g/mL and of the glycolic extract 1.004 g/mL).

ethanolic and glycolic extracts of *Chamomilla recutita* L., the results of which are shown in Table 5. Following hydrolysis, the amount of apigenin in methanolic, ethanolic and glycolic extracts increased ca. 9-, 11- and 22-fold, respectively, confirming the occurrence of apigenin glycosides in the original extracts. The concentrations of free and total apigenin in the methanolic extract of chamomile were 106 μg/g (0.01%, w/w) and 903 μg/g (0.1%, w/w) with respect to the dried flowers, respectively. The reported values for free and total apigenin in chamomile extracts are, however, somewhat higher. Tubaro *et al.* (1984) found 0.4% of total apigenin, whereas Zekovic *et al*. (1994) estimated that the total apigenin content was in the range 7–9% w/w for a number of different chamomile extracts. This divergence in measured apigenin levels might be explained by the fact that our extract was prepared with flowers harvested in the late stage of development at which point they consisted mainly of tubular florets which are richer in phenylpropanoids (Mulinacci *et al.*, 2000). Another possible explanation for the lower content of apigenin in our extracts is associated with the distinct climatic conditions under which the plants were cultivated. Since no other report on the phenolic content of chamomile flowers grown in Brazil could be found, no definitive conclusion about this aspect can be reached.

The results presented in this work have shown that CE is a feasible analytical technique for the quantitative determination of apigenin in chamomile extracts. With the proposed methodology, apigenin can be quantified in less than 8 min (total analysis time of 12 min including conditioning of the capillary column). This represents a significant improvement over the available HPLC methodologies which require from 25 to 50 min (Redaelli *et al*., 1981b; Maier *et al.*, 1991; Mauz *et al*., 1996; Scalia *et al*., 1999; Mulinacci *et al*., 2000). Moreover, CE presents a unique advantage for the analysis of herbal extracts in that there is no need for time-consuming protocols involved in sample clean-up and column conditioning. These advantages, along with the relatively low maintenance costs of CE, make the proposed methodology adequate for routine quality control of chamomile extracts.

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