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Separation of flavonoids and phenolic acids from propolis by capillary zone electrophoresis

The simultaneous determination of twelve different flavonoids, pinocembrin, acacetin, chrysin, rutin, catechin, naringenin, galangin, luteolin, kaempferol, apigenin, myricetin, and quercetin, two phenolic acids, cinnamic acid and caffeic acid, and one stilbene derivative, resveratrol, in propolis extracts used in medicine has been investigated by capillary zone electrophoresis (CZE). With a buffer constituted by sodium tetraborate 30 mM, pH 9.0, and 15 kV applied voltage, the 15 polyphenols were separated on an uncoated fused-silica capillary within 40 min using normal polarity. Under the experimental conditions used, a linear relationship was calculated between the CZE migration times and the molecular weight of polyphenols' expression of the increasing amount of their hydroxyl groups and polarity. Regression equations revealed a linear relationship (correlation coefficients > 0.97) between the peak area of each polyphenol species and their concentration, from 6 to 120 ng. The levels of analytes in three different propolis extracts, ethanolic, aqueous-ethanolic and aqueous-glycolic, used to prepare various commercial medicinal products, were determined. The aqueous-ethanolic propolis extract showed a great percentage of caffeic acid, galangin, quercetin, and chrysin, whilst the ethanolic preparation was composed of a great amount of resveratrol, chrysin, and caffeic acid. On the contrary, the aqueous-glycolic propolis preparation was composed of approx. 11% of caffeic acid and a low amount of the other identified flavonoids due to the presence of approx. 85% of nonidentified compounds. CZE represents a valuable method for the qualitative and quantitative assay of the most relevant polyphenol components of propolis, representing an alternative to obtain typical fingerprints of propolis and a reliable identification of a large number of propolis polyphenolic species.

Keywords: Capillary zone electrophoresis / Flavonoids / Phenolic acids / Polyphenols / Propolis
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1 Introduction

Research of the polyphenols (flavonoids and related phenolic acids) has been prompted by their visible beneficial effects on health, for example, their antimutagenic, anticarcenogenic, and antiatherogenic effects. Primarily flavonoids attracted great interest after they had been found to have effects in inhibiting the copper-catalyzed oxidation of low-density lipoprotein, inhibiting platelet clotting and arachidonate metabolism, reducing liver injury from peroxidized oil, and having cancer-chemopreventative properties [1]. Propolis is a resinous substance collected by honeybees from leaf buds and cracks in the bark of various plants, mainly from the poplar (*Populus*) genus and, to a lesser extent, beech, horsechestnut, birch, and conifer trees. Propolis has been used extensively in folk medicine for many years, and there is substantial evidence to indicate that propolis has antiseptic, antifungal, antibacterical, antiviral, anti-inflammatory, and antioxidant properties [2]. Current applications of propolis include over-the-counter preparations, mainly based on ethanolic extracts, for cold syndrome (upper respiratory tract infections, common cold, flu-like infection) as well as dermatological preparations useful in wound healing, treatment of boils, acne, herpes simplex and genitalis, and neurodermatitis [3].

In spite of possible differences in composition due to propolis-collecting bees that use resins from different plant sources, most propolis samples share considerable similarity in their overall chemical nature. Raw propolis is composed of 50% resin, composed of flavonoids and

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Abbreviations: Ac, acacetin; **Ap**, apigenin; **Ca**, catechin; **CaA**, caffeic acid; **Ch**, chrysin; **CiA**, cinnamic acid; **G**, galangin; **K**, kaempferol; **L**, luteolin; **M**, myricetin; **N**, naringenin; **P**, pinocembrin; **Q**, quercetin; **R**, rutin; **Re**, resveratrol

related phenolic acids and known as the polyphenolic fraction, 30% wax, 10% essential oils, 5% pollen, and 5% various organic compounds [4]. Propolis cannot be used as raw material, and it must be purified by extraction with solvents. This process should remove the inert material and preserve the polyphenolic fraction. A multistep extraction with ethanol is particularly suitable to obtain dewaxed propolis extracts rich in polyphenolic components [4]. These last compounds are considered to contribute more to the visible healing effects than the other propolis constituents. Flavonoids and phenolic acids, especially caffeates, are known for their antibacterial, antiviral, and antioxidant action [1].

Several methods have been developed to analyze polyphenols in various matrices: thin-layer chromatography, gas chromatography, high-performance liquid chromatography (HPLC), and HPLC-mass spectrometry are the most powerful analytical separation methods [5]. Due to its several advantages, such as the rapidity of the method, the small sample amounts required, and an extremely limited solvent waste, capillary electrophoresis (CE) has gained widespread interest as a favorable technique for the determination of pharmacologically interesting compounds in biological matrices, such as plants [6], leaves and flowers [7], herb medicines [8], tobacco [9], honey [10], tea [11], wine [12], human plasma, and urine. However, very few studies have reported on the characterization of flavonoids and phenolic acids from propolis by CE [13, 14]. This paper aims to gain new insight into the qualitative and quantitative separation of the polyphenolic component of propolis preparations in the form of ethanolic, aqueous-ethanolic, and aqueous-glycolic extracts, usually used for commercial pharmaceutical preparations, by means of the capillary zone electrophoresis (CZE) technique.

2 Materials and methods

2.1 Materials

The twelve different flavonoids, pinocembrin (P), acacetin (Ac), chrysin (Ch), rutin (R), catechin (Ca), naringenin (N), galangin (G), luteolin (L), kaempferol (K), apigenin (Ap), myricetin (M), and quercetin (Q), the two phenolic acids, cinnamic acid (CiA) and caffeic acid (CaA), and the stilbene derivative resveratrol (R) used in this study were purchased by Sigma (St. Louis, MO, USA). Their structures and some of their properties are illustrated in Fig. 1 and Table 1. Samples were prepared by dissolving the standard in ethanol at a concentration of 5 mg/mL and diluting 1:5 with the CZE running buffer to obtain a final concentration of 1 mg/mL. The different commercial propolis preparations were in the form of ethanolic, aqueous-ethanolic, and aqueous-glycolic extracts used to prepare various products such as oral sprays, tablets, and syrups. All these propolis preparations were procured from a local pharmacy. The different solutions were diluted 1:5 with the CZE running buffer, centrifuged at 5000 $\times g$ for 5 min and directly used for the analysis.

Table 1. Molecular weight and pK_a of the various polyphenols used in this study

Polyphenols	Molecular weight	pK_a
Resveratrol	228.24	9.14 ± 0.20
Pinocembrin	256.25	7.27 ± 0.20
Acacetin	284.26	6.88 ± 0.20
Chrysin	254.24	6.87 ± 0.20
Rutin	610.52	6.83 ± 0.60
Catechin	290.27	9.50 ± 0.10
Naringenin	272.25	7.58 ± 0.40
Cinnamic acid	148.16	3.88 ± 0.20
Galangin	270.24	6.91 ± 0.60
Luteolin	286.24	7.04 ± 0.40
Kaempferol	286.24	6.93 ± 0.60
Apigenin	270.24	7.08 ± 0.40
Myricetin	318.24	6.88 ± 0.60
Quercetin	302.24	6.89 ± 0.60
Caffeic acid	180.16	4.04 ± 0.40

The data were from SciFinder Scholar Database, Ver. 2002.1, by 2002 American Chemical Society.

2.2 Capillary electrophoresis

Capillary electrophoresis was performed on a Beckman CE instrument (P/ACE system 5000; Palo Alto, CA, USA) equipped with a UV detector set at 254 nm. Separation and analysis were carried out on an uncoated fused-silica capillary tube (50 μm ID, 70 cm total length, and 50 cm from the injection point to the detector) at 25°C. The operating buffer was constituted by sodium tetraborate, 30 mM, pH 9.0. The buffer was degassed by vacuum filtration through a 0.2 μm membrane filter, followed by agitation in an ultrasonic bath. Before each run, the capillary tube was washed with 0.1 M NaOH for 2 min and then conditioned with the operating buffer for 5 min. The samples to be analyzed were injected automatically, using the pressure injection mode. The injection volume can be calculated with the Poiseuille equation as proposed by the manufacturer, giving an estimated volume of 6 nL per second of injection time. The electrophoresis was performed at 15 kV (about 35 μA) using normal polarity. Peak areas were recorded and calculated using the Beckman software system Gold V810.

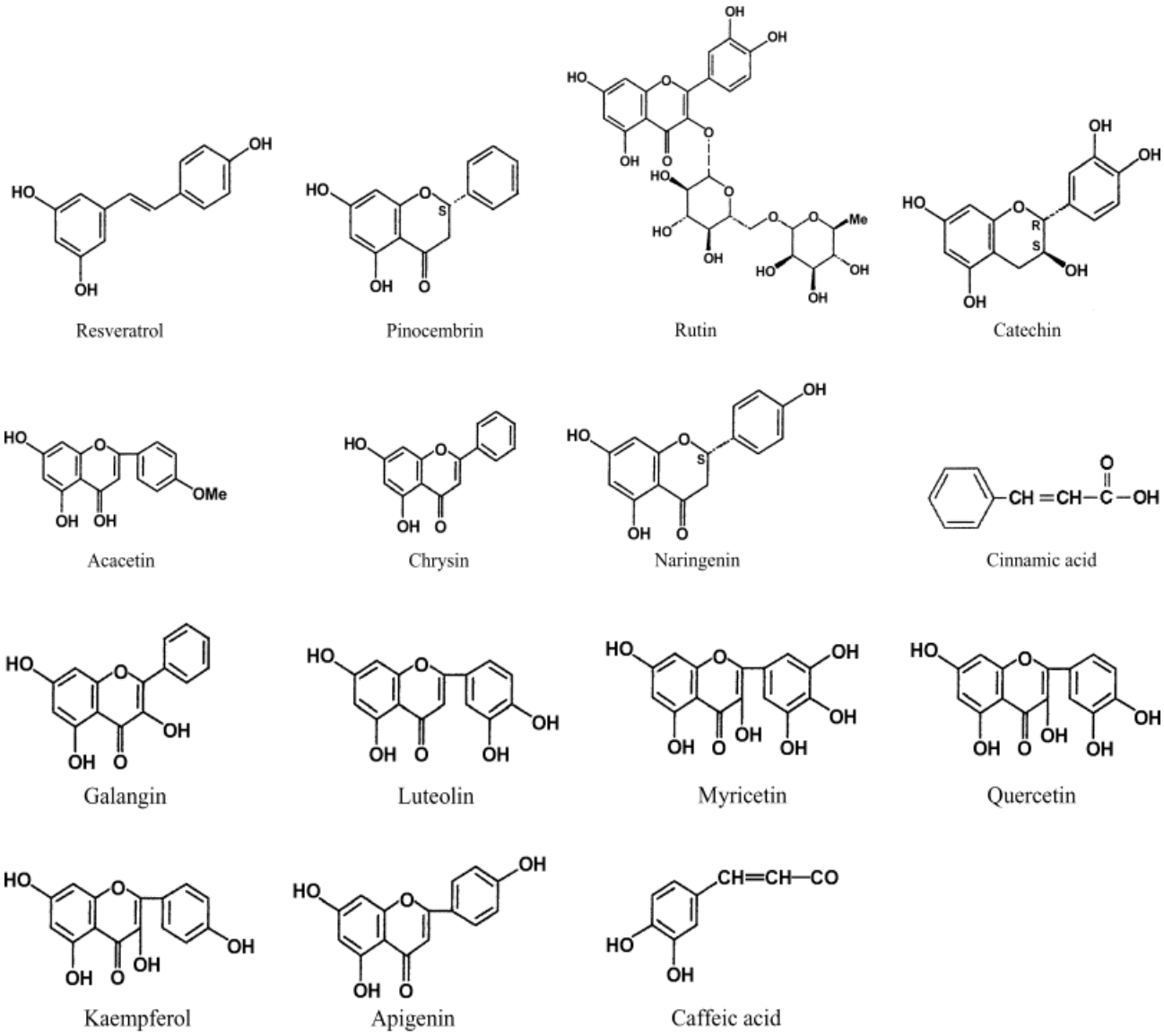

Figure 1. Structure and molecular weight of the 15 polyphenol compounds, 12 flavonoids, 2 phenolic acids, and 1 stilbene derivative separated by means of CZE. The structures and characteristics of polyphenols were from SciFinder Scholar Database, Ver. 2002.1, by 2002 American Chemical Society.

2.3 Linearity and detection sensitivity

In order to test the linearity of the detector response for CZE, the different polyphenol solutions prepared as reported above were injected at increasing times, from 1 to 20 s corresponding to an injection amount from 6 to 120 ng. The calibration graphs were constructed by plotting the peak areas of flavonoids and phenolic acids against their concentration. The detection limits were estimated as the quantity of the molecules producing a peak height signal twice the baseline noise.

3 Results

Figure 2 demonstrates, as an example, the satisfactory resolution obtained for 11 of the 15 polyphenols used in this study. It is necessary to consider that the migration times for the different molecular species changed according to a percentage calculated to be approx. 20%. However, the migration times were found to be more constant when rinsing of the capillary with NaOH was used between each separation. As evident from Fig. 2, Ch, R, and Ca (migration times of 13.94, 14.87, and 15.26), K

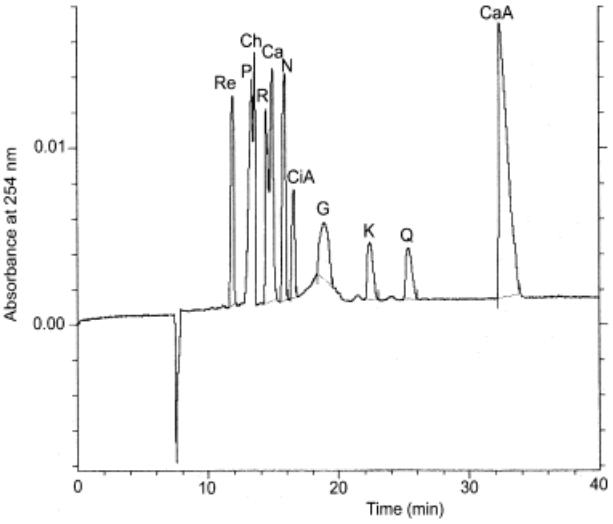

Figure 2. CZE electropherograms of 11 flavonoid and phenolic acid standards.

and Ap (migration times of 22.98 and 23.28), and M and Q (migration times of 25.87 and 26.50) show very close migration time values, making the CZE separation difficult to explain. As a consequence, the identification of the polyphenols in propolis extracts was performed by adding single species of standard to each sample.

The sensitivity and linearity of the CZE method for the separation of polyphenols were tested with the use of commercial standards at various concentrations. A linear relationship (correlation coefficients > 0.97, Table 2) was found for the main species identified in the propolis extracts over a wide range of concentrations, from approx. 6 to 120 ng. The CZE separation produced a great detection sensitivity, lower than about 10 ng. As illustrated in Table 2, an increasing amount of flavonoids and of the stilbene derivative R produce approx. the same detector response at 254 nm resulting in equal calibration curves. On the contrary, CaA has a greater absorbance capacity at 254 nm and this was also observed for the other phenolic acid, CiA. The precision of the method was determined by three repeated determinations of the various polyphenols. When 90 ng of the standard were measured, the relative standard deviations were always calculated to be lower than approx. 8% (Table 2).

Figures 3A–C show the electropherograms of the acqueous-ethanolic, ethanolic, and aqueous-glycolic propolis extracts, respectively. By adding to these extracts single species of standard, as previously reported, several kinds of polyphenols were identified and quantified by using the calibration curves illustrated in Table 2. Of the 15 standards used in this study, only luteolin, apigenin,

Table 2. Calibration curves for the analysis of the various polyphenol species showing detector response, expressed as peak area, as a function of their concentration in ng

Polyphenols	Equation	r^2	CV%
Resveratrol	$y = 0.146x + 1.316$	0.975	5.7
Pinocembrin	$y = 0.130x + 0.024$	0.998	6.8
Acacetin	$y = 0.137x - 1.835$	0.977	7.3
Chrysin	$y = 0.178x + 1.147$	0.982	4.9
Rutin	$y = 0.120x + 0.033$	0.987	7.2
Catechin	$y = 0.160x - 0.552$	0.994	6.8
Naringenin	$y = 0.133x + 1.216$	0.992	5.9
Cinnamic acid	$y = 0.659x + 2.004$	0.988	6.8
Galangin	$y = 0.177x + 0.513$	0.995	7.5
Luteolin	$y = 0.114x - 1.635$	0.946	7.8
Kaempferol	$y = 0.111x + 0.913$	0.979	6.5
Apigenin	$y = 0.154x + 0.426$	0.988	7.3
Myricetin	$y = 0.132x + 0.345$	0.984	7.6
Quercetin	$y = 0.180x + 0.771$	0.995	8.4
Caffeic acid	$y = 0.679x + 2.117$	0.998	8.8

Calibration curves performed in CZE from 6 to 120 ng. The equations and the correlation coefficients (r^2) are reported. The coefficient of variations (CV%) determined at 90 ng of the standards is also shown.

and myricetin were not detected in the three propolis extracts (Table 3). As is also evident from Fig. 3B, the electropherogram of the ethanolic extract is quite different from those of the aqueous-ethanolic and aqueous-glycolic samples, in particular in the lower migration times compared to the other two preparations. By using internal standards, the single flavonoid and phenolic acid species were identified, and the shift in the migration times was ascribed to the presence in this preparation of a greater amount of ethanol than in the other two samples. In fact, organic solvents such as methanol and acetonitrile have been used in some CZE separations of flavonoids to modify the electrophoretic migration depending on the molecular species [12].

Table 3 illustrates the percentage of the single polyphenol species calculated using the specific calibration curves for the three extracts. The aqueous-ethanolic propolis extract shows a great percentage of CaA (approx. 40%), G (18%), Q (8.7%), and Ch (7.8%). On the contrary, the ethanolic preparation is composed of a large quantity of Re (21.9%), Ch (18.7%), and CaA (approx. 15%). As can be observed, great differences in the composition of flavonoids and phenolic acids can be observed for the two extracts. The high content of the stilbene derivative Re in the ethanolic sample requires further studies and it is actually under investigation by using liquid chromatography – electrospray ionization – mass spectroscopy

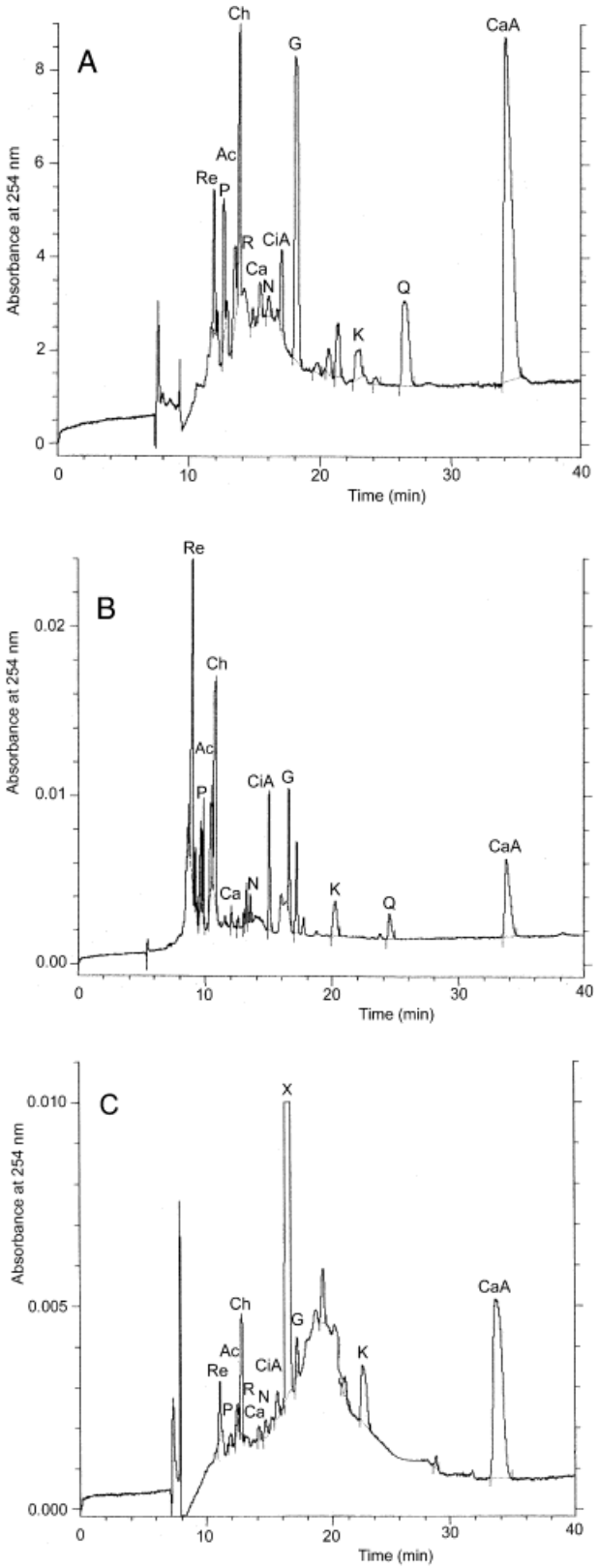

Figure 3. CZE electropherograms of (A) propolis acqueous-ethanolic extract, (B) propolis ethanolic extract, and (C) propolis aqueous-glycolic extract.

(LC-ESI-MS). The aqueous-glycolic propolis preparation is composed of approx. 11% of CaA and of a very low amount of the other identified flavonoids. However, this extract was found to be mainly constituted by approx. 85% of nonidentified compounds, in particular a molecular species having a migration time of approx. 17.50 and marked in Fig. 3C as x. The percentage of the nonidentified molecular species was calculated to be approx. 6% for the aqueous-ethanolic extract and 12.4% for the ethanolic propolis preparation (Table 3). The CZE analysis of various aqueous-ethanolic extracts, and several ethanolic and aqueous-glycolic propolis preparations, always showed the same qualitative and quantitative pattern of the polyphenolic fraction creating a reproducible fingerprint depending on the nature of the extraction solvent (not shown).

Table 3. Polyphenols identified and quantified in the three propolis extracts used in medicine

Polyphenols	Acqueous-ethanolic	Ethanolic	Acqueous-glycolic
Resveratrol	3.8	21.9	0.6
Pinocembrin	4.0	3.4	0.2
Acacetin	3.4	6.6	0.3
Chrysin	7.8	18.7	1.6
Rutin	0.6	n.d.	Trace
Catechin	1.3	0.4	0.2
Naringenin	1.0	1.6	0.3
Cinnamic acid	3.1	7.0	0.4
Galangin	18.1	5.8	0.4
Luteolin	n.d.	n.d.	n.d.
Kaempferol	2.5	4.8	1.1
Apigenin	n.d.	n.d.	n.d.
Myricetin	n.d.	n.d.	n.d.
Quercetin	8.7	2.8	n.d.
Caffeic acid	40.1	14.9	10.7
Not identified	5.6	12.4	84.2

n.d., not detected

The results are the mean of three different experiments for each extract. The amount of each polyphenolic species was calculated by using specific calibration curves (see Table 2) and reported as percentage being 100% the sum of known polyphenolic compounds and unidentified species. The percentage of not identified molecular species was calculated by considering the calibration equation of galangin due to the very close regression trend of the different flavonoids (see also Figs. 3A–C).

4 Discussion

Borate buffers with pH 8–11 and a concentration of 25 – 200 mM are commonly used, as borate can form complexes with orthodihydroxyl groups on the flavonoid

nucleus and therefore facilitate the separation [6]. In this study, we used a buffer constituted by sodium tetraborate 30 mM, pH 9.0, and the separation of 12 flavonoids, 2 phenolic acids, and 1 stilbene derivative standard (see Fig. 1) was obtained in approx. 40 min on an uncoated fused-silica capillary tube (50 μm ID, 70 cm total length, and 50 cm from the injection point to the detector). With this technique, qualitative and quantitative analysis of propolis polyphenol extracts from different solvents (*i.e.*, acqueous-ethanolic, ethanolic, and aqueous-glycolic) was performed. No pretreatment of the samples was performed as the different commercial propolis preparations are in the form of ethanolic, aqueous-ethanolic, and aqueous-glycolic extracts and are used to prepare various products such as oral sprays, tablets, and syrups. As a consequence, a rapid quantitative and qualitative CZE separation of these products is of interest.

Specific complexation between borate ion and certain analytes containing hydroxyl groups results in negatively charged borate complexes. The borate buffer, under basic experimental conditions in which the polyphenols are negatively charged, could effect the separation either based on charge-to-mass ratios of the deprotonated polyphenols or through borate-phenol association [6, 15]. As a consequence, the CZE migration times of the polyphenol compounds increase with their polarity. In fact, considering flavonoids having the same molecular nucleus structure, *i.e.*, benzopyran-4-one, 5,7-dihydroxy-2-phenyl, a linear relationship (Fig. 4) with a correlation coefficient greater than approx. 0.8 was calculated between the CZE migration times and the molecular weight of flavonoids expression of the increasing amount of their hydroxyl groups and polarity (see Fig. 1). Furthermore, the migration times of CZE were also evident for the two phenolic acids separated in this study, with the most polar CaA, dihydroxycinnamic acid, showing a migration time greater than that of the CiA (approx. double under the experimental conditions adopted).

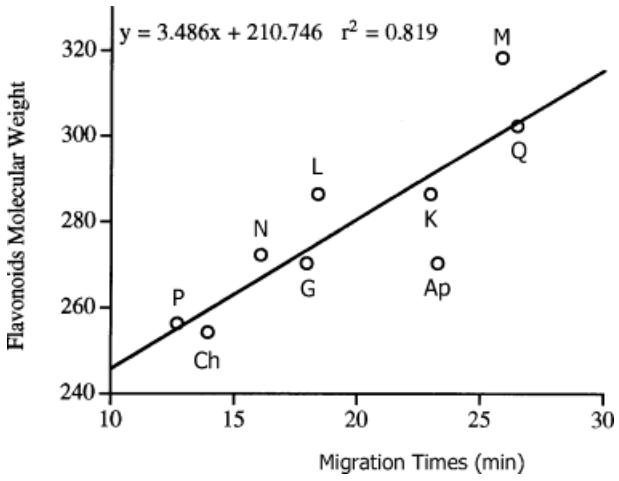

Figure 4. Correlation between the CZE migration times and the molecular weight of flavonoids having the same molecular nucleus structure benzopyran-4-one, 5,7-dihydroxy-2-phenyl, expression of the increasing amount of their hydroxyl groups and polarity (see Fig. 1 for structures). The equation and the correlation coefficient are reported. The species corresponding to each point are illustrated.

The CZE technique was applied to separate and quantify polyphenols in propolis extracts used in medicine. Depending on the extraction solvent, different patterns of polyphenol compounds were separated by CZE. In particular, the aqueous-ethanolic propolis extract is abundant in the most polar flavonoids, such as CaA, G, and Q, while the ethanolic preparation shows a great percentage of the less polar flavonoids, such as Re and Ch (see Fig. 1). Obviously, the ethanol alone in the extraction solvent is unable to extract the most polar component of polyphenols, contrary to the aqueous-ethanolic solvent. The aqueous-glycolic propolis preparation was found to be mainly composed of approx. 11% of caffeic acid and of a very low amount of the other identified flavonoids, as this extract was found to be mainly constituted by approx. 85% of nonidentified compounds.

In a study conducted by means of HPLC, several acqueous-ethanolic, ethanolic and aqueous-glycolic propolis extracts were analyzed for their flavonoid component, and the most abundant species were found to be G, P, Ch, CaA, Q, K, and N, yet differing in the content of specific components [4]. As reported in Table 3, the major flavonoid species calculated in this study are CaA, Ch, G, Q, K, P, and Re, even if also in this case quantitative differences of specific species are calculated depending on the kind of extract. Clearly, a good correspondence between HPLC and CZE techniques may be considered. Furthermore, several nonidentified molecular species were calculated to be approx. 6% for the aqueous-ethanolic extract and 12.4% for the ethanolic propolis extract (Table 3). According to Pietta *et al.* [4], these species are probably derivatives of the most representative polyphenols, such as CaA-derivatives and Q-derivatives.

Hilhorst *et al*. [13] described a CZE separation of water extracts of propolis but no analysis was performed on aqueous-ethanolic, ethanolic, or aqueous-glycolic preparations generally considered the most suitable extraction solvents to recover high content of flavonoids and, at the same time, to minimize the extraction of hydrophobic wax-like compounds [4, 16]. As a consequence, a propolis extract very rich in phenolic acids was investigated [13]. By performing a MEKC separation of propolis alcoholic extracts, the same authors [13] and Fontana *et*

al. [14] found P, Ch, and G to be the flavonoids at the highest concentration, in good agreement with the present study. Furthermore, Bankova *et al*. [16, 17] also found that P, G, and Ch are the main flavonoids in other propolis samples.

To our knowledge, this is the first paper describing CZE analysis of propolis extracts performed by various solvents generally used to prepare various pharmaceutical products. On the basis of the results of this study, it may be concluded that CZE represents a valuable method for the qualitative and quantitative assay of the most relevant components of propolis. Direct CZE analysis constitutes an alternative to obtain typical fingerprints of propolis and a reliable identification of a large number of propolis polyphenolic components.

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