Potential of Capillary Electrophoresis for the Profiling of Propolis

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Summary

The usefulness of capillary electrophoresis (CE) with diode array detection for the profiling of Propolis, a hive product, is investigated. Water extracts of Propolis were analyzed with both capillary zone electrophoresis (CZE) at pH 7.0 and 9.3, and micellar electrokinetic chromatography (MEKC) with sodium dodecyl sulfate at pH 9.3. Characteristic profiles were obtained and several organic acids and preservatives could be identified by means of library comparison of the recorded UV spectra combined with addition of reference compounds to the extracts. The selectivity of the CZE and MEKC system differed considerably but the information obtained with both methods was similar. The dry residues of the water extraction were extracted with ethanol-water (70 : 30, *v/v*) and analyzed with the MEKC system to enable the separation of the more hydrophobic constituents of the Propolis samples. Complex profiles containing various well separated peaks were obtained allowing the identification of some interesting flavonoids. On the basis of the recorded CZE and MEKC profiles, the Propolis samples could be divided into two clearly different groups which are probably from a different origin.

1 Introduction

Propolis is a resinous hive product collected by bees. It is known to possess antibacterial, fungicidal, local anaesthetic, antiulcer, immunostimulating, hypotensive, and cytostatic properties [1–6]. Propolis is used in folk medicine and has recently found applications in the clinical area [7, 8]. Propolis is a wax-like material containing a variety of compounds. Among these are phenolics such as flavonoids and phenolic acids which are the biologically most active constituents [8]. The concentrations of phenolic compounds in Propolis may vary substantially according to the origin of the samples. Such differences are likely to affect its clinical properties [9]. Therefore, analytical methods are needed to generate profiles capable of distinguishing Propolis from different origins and to determine the biologically most important constituents. The origin of plants and honey has been determined by measuring the profiles of their flavonoid and phenolic acid constituents with liquid chromatography (LC) [10, 11]. Bankova *et al.* analyzed phenolic compounds in Propolis by LC [12] and gas chromatography (GC) [8]. However, LC is limited in separation power and GC can only be applied for a limited number of flavonoids and phenolic acids due to their thermolability [9].

Capillary electrophoresis (CE) may be an interesting alternative for the analysis of Propolis. CE offers high efficiency, speed and low consumption of chemicals and is becoming increasingly popular in the pharmaceutical and related fields. In CE different modes of operation can be distinguished. Capillary zone electrophoresis (CZE) is based on differences in the electrophoretic mobility of compounds caused by their charge and size. Micellar electrokinetic chromatography (MEKC), in which surfactants are added to the separation buffer, is also capable of separating neutral compounds. Using CZE, Seitz *et al.*[13] demonstrated the simultaneous determination of some phenolic acids and flavonoids in a standard mixture. McGhie [14] used CZE for the analysis of flavonoids in sugarcane. Since flavonoids are only weakly acidic, their separation requires a buffer of pH > 10 to be successful. Chi *et al.* [9] demonstrated the determination of flavonoids and phenolic acids in Propolis by CZE using a buffer with pH 10.1. However, important flavonoids like myricetin and quercetin, may decompose in such alkaline media [15]. Therefore, preferably MEKC is used for the determination of flavonoids in natural samples. With MEKC, flavonoids have been identified in honey [15] and plant extracts [16–22] using sodium dodecyl sulfate (SDS) as well as cetyltrimethylammonium bromide as surfactant, but the MEKC analysis of Propolis has not been reported before. Ferreres *et al.* showed that the characterization of honey could be based on either the phenolic acids profile measured by HPLC [23], or the flavonoids profile determined by MEKC [15]. They pointed out that simultaneous determination of phenolic acids and flavonoids caused mutual interference. When phenolic acids were analyzed, a clean up step was necessary in order to remove disturbing UV-absorbing substances, such as the flavonoids.

In the present study Propolis is analyzed by CZE and MEKC and a simple sample pretreatment procedure is used to avoid interference. First, the Propolis samples are extracted with water to isolate the charged and relatively polar constituents, such as the phenolic acids. The extracts are subsequently analyzed with CZE using separation buffers with pH 7.0 and 9.3, and with MEKC at pH 9.3 using SDS as surfactant. The water insoluble residues are extracted with an ethanol-water mixture to isolate the flavonoids. These extracts are analyzed with MEKC only, because most of the extracted compounds are expected to be neutral. The profiles obtained under the different experimental conditions have been compared and, if possible, the present phenolic acids and flavonoids have been identified.

2 Experimental

2.1 Samples

The water extracts of ten Propolis samples, as well as their water-insoluble residues, were obtained from Propharma A/S (Havdrup, Denmark). The water extracts were filtered over a FP 030/3 (Schleicher and Schuell, Dassel, Germany) 0.2 µm filter and diluted ten times before injection. The water insoluble residues were freeze dried, pulverized and homogenized, and then extracted by the procedure described by Chi *et al.* [9]: 500 mg of each residue was sonicated in 25 ml ethanol-water (70 : 30 *v/v*) for 30 minutes. The suspension was then passed through a paper filter (Schleicher and Schuell, Dassel, Germany) into a 50 ml volumetric flask. Before injection, the alcoholic extracts were filtered through a FP 030/3 filter and diluted five times with the extraction solvent.

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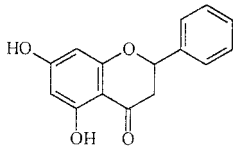
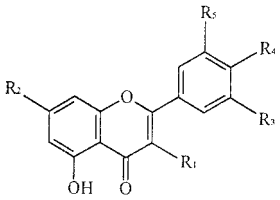
2.2 Chemicals

Ethanol and acetonitrile (ACN) were purchased from Labscan (Dublin, Ireland), potassium dihydrogenphosphate, boric acid, phosphoric acid, and sodium dodecyl sulfate (SDS) from Merck (Darmstadt, Germany). The phenolic acid reference compounds were purchased from Sigma (St Louis, MO, USA). The flavonoids (**Table 1**) myricetin, luteolin, kaempferol, kaempferid, rhamnetin and isorhamnetin were from Carl Roth (Karlsruhe, Germany), chrysin, acacetin, pinocembrin, galangin and tectochrysin from Extrasynthese (Genay, France), quercetin from Fluka (Buchs, Switzerland) and apigenin from ICN (Zoetermeer, The Netherlands). All chemicals were of analytical grade. Deionized water was obtained by an Elga ultra pure water system (Salm en Kipp BV, The Netherlands). For the CZE experiments two buffers were prepared as background electrolyte: phosphate (25 mM, pH 7.0) and borate (25 mM, pH 9.3). All buffers were adjusted to the proper pH by addition of 2.0 M sodium hydroxide. The buffer used in the MEKC experiment contained borate (25 mM, pH 9.3) with 50 mM SDS and 10% (*v/v*) acetonitrile. The separation buffers were filtered over a 0.45 µm membrane filter (Schleicher and Schuell, Dassel, Germany) before use.

Table 1. Structure of the reference flavonoids.



Pinocembrin



Flavonoid	R_1	R_2	R_3	R_4	R_5
Myricetin	OH	OH	OH	OH	OH
Luteolin		OH	OH	OH	
Quercetin	OH	OH	OH	OH	
Apigenin		OH		OH	
Kaempferol	OH	OH		OH	
Iso-rhamnetin	OH	OH	OCH_3	OH	
Rhamnetin	OH	OCH_3	OH	OH	
Chrysin		OH			
Acacetin		OH		OCH_3	
Galangin	OH	OH			
Kaempferid	OH	OH		OCH_3	
Tectochrysin		OCH_3			

2.3 CE System

The experiments were performed with a HP^{3D} Capillary Electrophoresis system (Hewlett Packard, Waldbronn, Germany) equipped with an on-column diode array detector (DAD). Uncoated fused silica capillaries of 64.5 cm × 50 µm i.d. with an effective length of 56.0 cm were used. The capillaries contained a bubble detection cell which extends the capillary diameter, and thus the optical path length, to 150 µm at the detection window. The capillaries were used for either the CZE or the MEKC experiments. Before use, the capillaries were rinsed with 1 M NaOH (15 min), followed by deionized water (15 min) and separation buffer (30 min). Between runs, the capillary was flushed with the separation buffer for two minutes. The capillary was thermostated to 25 °C and samples were hydrodynamically injected by applying a pressure of 50 mbar for 2 seconds. Electrophoresis was performed at a constant voltage of 30 kV. The data were collected using HP Chemstation software version 04.02. The electropherograms were interpreted at 200 nm.

2.4 Identification Procedure

The reference solutions of the flavonoids and phenolic acids were analyzed under the same conditions as the samples, and their UV spectrum was added to the spectral library. Because of the gradual change of the electroosmotic flow (EOF), migration-time data were not included in the library. For identification of an unknown peak, the software compares the spectrum of the peak with each reference spectrum in the library and calculates a match factor (0–1 000) representing the degree of spectral similarity. The larger the value of the factor, the better the match of the spectra. When the reference compound with the best similarity had a match factor above 950, the compound was added to the sample and the mixture was analyzed again. The peak areas with and without the addition of the reference compound were compared and in case of an increase the component was considered as identified.

3 Results and Discussion

3.1 CZE of Water Extracts

First the potential of CZE for the profiling of the water extracts was investigated. The extracts are likely to contain phenolic acids which have a good solubility in water. CZE experiments were carried out at pH 7.0 and 9.3 so that the phenolic acids, which have p*K*a values of about 5, are charged. On the basis of the electrophoretic profiles obtained at pH 7, the Propolis extracts could be divided into two distinct groups of extracts 1–7 and extracts 8–10, respectively. Within one group the electropherograms showed great similarity with respect to number, position and area of the peaks. The patterns of the two groups are clearly different, as can be observed in **Figure 1** which shows two representative electropherograms of each group. Due to capillary wall effects and buffer depletion, the EOF gradually changes during the analyses of the ten samples. This explains the shift in migration times which is particularly observable for the late migrating compounds. Using the spectral library and addition of reference compounds, 3,4-dimethoxycinnamic acid (peak 2), *p*-coumaric acid (peak 4), cinnamic acid (peak 5) and benzoic acid (peak 6) could be identified in the samples 1–7. Library search of the spectrum of peak 3 resulted in a match factor above 950 for both ferulic acid and 3,4-dihydroxycinnamic acid. However, addition of these reference compounds revealed that their migration times did not match the migration time of peak 3. Probably, the unknown compound is a phenolic acid which seems structurally related to cinnamic acid. Obviously, all uncharged components present in the sample migrate over the time the EOF requires to reach the detector (t_{EOF}). The spectrum of the peak at t_{EOF} showed good similarity with the spectra of both methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate. Such esters are not naturally occurring components of Propolis but have been added to the water extracts for preservation pur-

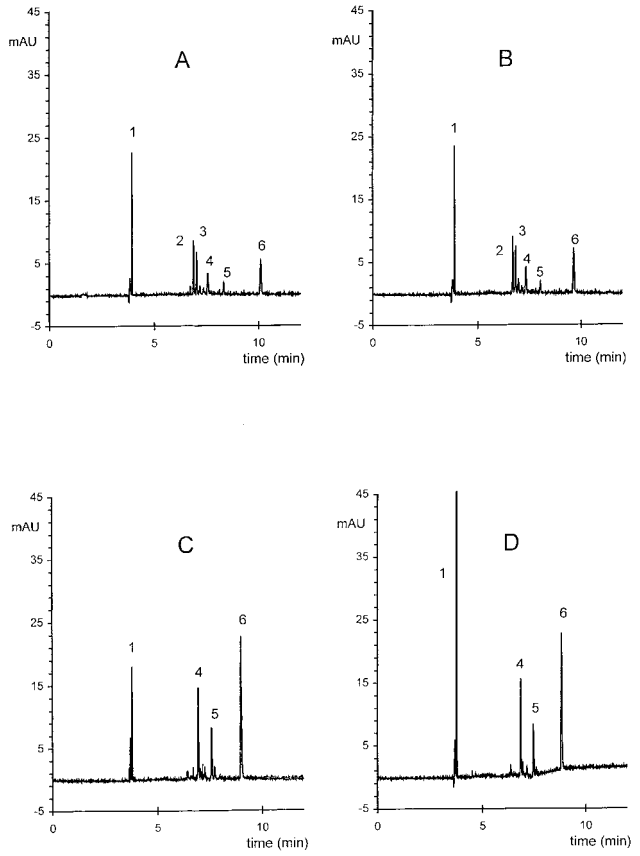


Figure 1. Electropherograms of Propolis water extracts analyzed by CZE at pH 7.0. (A) extract 1, (B) extract 5, (C) extract 9 and (D) extract 10. Peaks: 1 = EOF peak (see text); 2 = 3,4-dimethoxycinnamic acid; 3 = not identified; 4 = *p*-coumaric acid; 5 = cinnamic acid, 6 = benzoic acid.

poses. Since all uncharged components migrate with the EOF, addition of reference compounds could not be used as identification method. In the samples 8–10, only *p*-coumaric acid (peak 4), cinnamic acid (peak 5) and benzoic acid (peak 6) could be identified. Their concentration appears to be a factor 3–5 higher than in the samples 1–7. The cinnamic acid related compound and 3,4-dimethoxycinnamic acid were not detected in the 8–10 samples.

The migration order of the organic acids can be explained by their radius. Since the pK_a values of the acids are *ca.* 5 they are all completely charged. Obviously, the radius of the acids is the largest for 3,4-dimethoxycinnamic acid and therefore this compound migrates first, while benzoic acid has the smallest radius and migrates last.

When the water extracts were analyzed with a separation buffer of pH 9.3 also efficient separations were obtained. The profiles were characteristic and the distinction in two groups (1–7 and 8–10) could be made again (**Figure 2**). In the samples the phenolic acids 3,4-dimethoxycinnamic (samples 1–7 only), cinnamic, benzoic and *p*-coumaric acid could again be identified. However, the migration order of the acids is different compared to CZE at pH 7.0. The shift of *p*-coumaric acid, for example, can be explained by the pH change. At pH 9.3 *p*-coumaric acid (peak 4), which contains both a carboxyl and a hydroxyl group

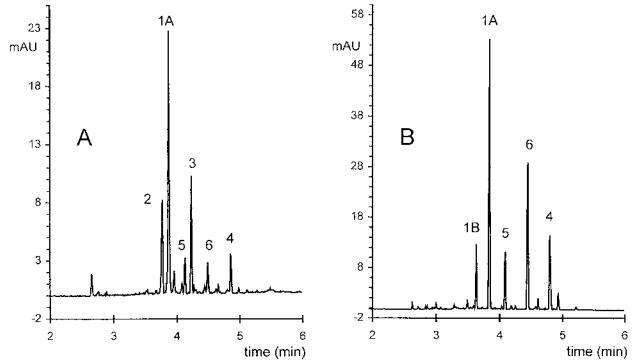


Figure 2. Electropherograms of Propolis water extracts analyzed by CZE at pH 9.3. (A) extract 1, (B) extract 10. Peaks: 1A = methyl *p*-hydroxybenzoate; peak 1B = propyl *p*-hydroxybenzoate; further peak denotation as in Figure 1.

(pK_{a1} = 4.64, pK_{a2} = 9.45) [24], is more negatively charged than 3,4-dimethoxycinnamic acid (peak 2), cinnamic acid (peak 5) and benzoic acid (peak 6) which are all monoprotic. Therefore, *p*-coumaric acid has the largest migration time. This pH dependency of migration times of cinnamic acid analogues has also been observed by Fujiware *et al.* [25]. At pH 9.3, compared to CZE at pH 7.0, only a relatively small peak is present at t_{EOF}. However, an extra peak (peak 1A, Figure 2 A) appeared and the spectrum of this peak showed good similarity with the library spectrum of methyl *p*-hydroxybenzoate and propyl *p*-hydroxy benzoate. Addition of reference compounds revealed the unknown peak to be methyl *p*-hydroxybenzoate. During the CZE analysis at pH 9.3 of the extracts 8–10 again no significant peak migrated with the EOF, however, now two extra peaks (1A and 1B, Figure 2B) appeared with respect to CZE at pH 7.0. These could be identified as methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate, respectively. Apparently, the hydroxy group of the esters is (partly) deprotonated at pH 9.3 resulting in a electrophoretic mobility which enables their separation. Methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate are likely to have a similar pK_a and, therefore, the largest of the two, propyl *p*-hydroxybenzoate, elutes first. The profiles recorded with CZE at pH 9.3 confirm the presence of the unidentified compound (peak 3) in the samples 1–7; it now migrates at 4.2 min.

3.2 MEKC of Water Extracts

In the CZE experiments using a buffer of pH 9.3, no significant peak was found at t_{EOF}. Therefore, it seems that at this pH neutral components are only present in a negligible amount. However, the presence of neutral compounds in the extracts of various samples cannot be excluded beforehand. It is therefore interesting to investigate whether the profiling of the water extracts can be achieved with MEKC, because MEKC has the potential to separate neutral components as well. Naturally, the separation of the charged analytes should be maintained in the MEKC system. In a previous paper [26] we have demonstrated that a MEKC system containing 25 mM borate (pH 9.3) 50 mM SDS and 10% ACN provides a large migration window (*i.e.* relatively high resolution) with reasonable analysis times for both charged and neutral compounds. For the water extracts of Propolis, this MEKC system also shows favourable results (**Figure 3**). As with

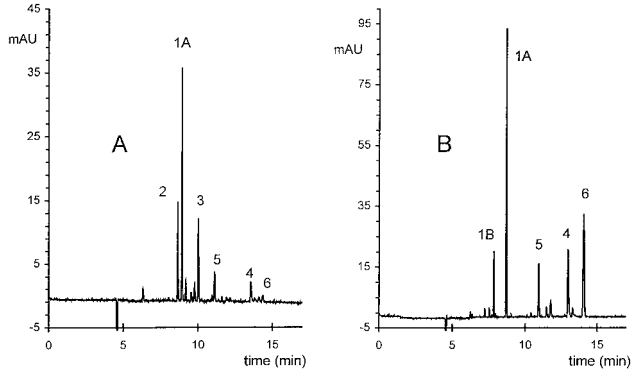


Figure 3. Electropherograms of Propolis water extracts analyzed by MEKC at pH 9.3. (A) extract 1, (B) extract 10. Peak denotation, see Figure 2.

CZE at pH 9.3, the MEKC system is capable of separating all components, allowing the identification of the phenolic acids and esters. Interestingly, the migration order in the MEKC experiments differs from the peak order in the CZE experiments at the same pH value, indicating a selective interaction of the analytes with the micelles. For example, the peak order of *p*-coumaric acid (peak 4) and benzoic acid (peak 6) is reversed. The same is true for the unidentified cinnamic acid analogue (peak 3) and cinnamic acid (peak 5).

3.3 MEKC of Alcoholic Extracts

The possibility of profiling the Propolis samples by the analysis of the alcoholic extracts of the residues (see Experimental section) was also investigated. Such an analysis preferably should allow the determination of the important flavonoids which are not present in the water extracts. In principle, flavonoids can be extracted effectively from Propolis samples using extraction solvents such as acetone, ethanol or methanol [9]. However, Bankova *et al.* [8] showed that in order to recover the flavonoids, but at the same time minimize the extraction of hydrophobic wax-like compounds, ethanol-water (70 : 30 *v/v*) was the most suitable extraction solvent. Because a variety of neutral compounds can be expected in the alcoholic extracts, the MEKC system described above was applied.

The alcoholic extracts indeed appear to contain a large number of compounds, resulting in a complex but characteristic MEKC profile (**Figure 4**). On the basis of the recorded profiles, again, a clear distinction could be made between the Propolis samples 1–7 and 8–10, whereas within one group the profiles showed great similarity. Most peaks are well distributed over the migration window, resulting in a good resolution. The more hydrophobic compounds migrate at the time the micelles need to reach the detector, causing no interference with the region where some important flavonoids migrate. Although the electrokinetic chromatograms show sufficient peaks for a useful profiling, separation of the compounds in the broad band should be possible by increasing the amount of acetonitrile or by decreasing the SDS concentration. However, this would also result in a decrease in resolution of the earlier migrating peaks, including the flavonoids. The addition of bile salts [27] or cyclodextrins [28] is another option.

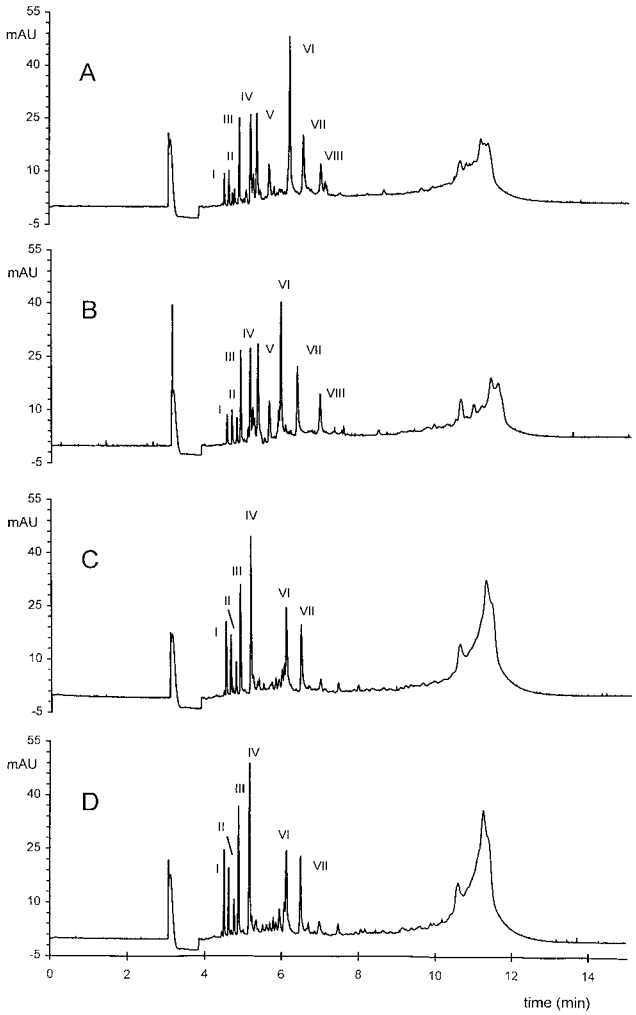


Figure 4. Electropherograms of the alcoholic Propolis extracts analyzed by MEKC at pH 9.3. (A) extract 1, (B) extract 5, (C) extract 9, (D) extract 10. Peaks: I, II, III = not identified; IV = pinocembrin; V = not identified; VI = chrysine; VII = galangin; VIII = not identified.

None of the peaks could be recognized as a phenolic acid by the library. Despite the large number of eluting compounds, most peaks in the 4–8 min region are well resolved allowing the reliable identification of the flavonoids pinocembrin (peak IV), chrysin (peak VI) and galangin (peak VII) in all samples. The identification was based on both spectral search and addition of reference compounds. In samples 1–7, chrysin is the flavonoid with the highest concentration and from comparison of peak heights it can be concluded that the chrysin concentration is about two times higher than in samples 8–10. In samples 8–10, pinocembrin is the highest peak and is about twice that of the pinocembrin peak in the samples 1–7. Besides the differences in flavonoid concentrations between samples 1–7 and 8–10, the most striking differences are peak V and VIII (Figure 4) which are present in 1–7 but very low or absent in 8–10, and peaks I and II which are considerably higher in samples 8–10. For Bulgarian Propolis, Bankova *et al.* also found that pinocembrin and galangin, which are responsible for the antibacterial properties of Propolis [3], and chrysin are the main flavonoids [12]. Using GC, Bankova also confirmed the presence of galangin and pinocembrin in other Propolis samples [8].

4 Conclusions

CZE and MEKC with diode array detection are very useful techniques for the profiling of natural and related products. This is demonstrated by the analysis of aqueous and alcoholic extracts of the hive product Propolis. With both CZE (borate pH 9.3) and MEKC (50 mM SDS, 10% ACN, borate pH 9.3) good separations are obtained for all components present in the aqueous extracts. On the basis of the obtained profiles, the samples could be divided into two distinct groups and several interesting phenolic acids were successfully identified in all samples. The presence of these compounds can be demonstrated down to the low ppm range in the aqueous extracts. Because MEKC has the potential to separate neutral compounds as well, the MEKC system seems to be the best choice for the analysis of aqueous Propolis extracts.

MEKC analysis of the alcoholic extracts revealed electropherograms with a large number of well separated peaks. Due to the combination of a relatively polar extraction solvent (ethanol-water, 70 : 30 *v/v*) and the applied MEKC system (50 mM SDS, 10% ACN) the very hydrophobic substances were either not extracted or did not interfere with the analysis since they migrated with the same velocity as the micelles. As a result, most extracted components are well resolved and the important flavonoids pinocembrin, galangin, and chrysin could be identified easily and reliably for concentrations higher than about 1 ppm in the Propolis residues. Interestingly, with the characteristic profiles obtained for the alcoholic extracts, exactly the same distinction into groups of Propolis samples could be made as with CZE. In other words, the character (*i.e.* the origin) of the Propolis samples is reflected in both the aqueous and alcoholic extracts and the used CE techniques show very good potential for the characterization of Propolis samples.

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References

[1] V.S. Bankova, S.S. Popov, N.L. Marekov, *Phytochemistry* **1989**, *28*, 871.

[2] J. Focht, S.H. Hansen, J.V. Nielsen, A. Van den Berg-Segers, R. Riezler, *Arzneim. Forschung* **1993**, *43*, 921.

[3] S. Pepeljnjak, I. Jalsenjak, D. Maysinger, *Pharmazie* **1985**, *40*, 122.

[4] J. Serkedjieva, N. Manolova, V. Bankova, *J. Nat. Prod.* **1992**, *55*, 294.

[5] J. Metzner, E.M. Schneidewind, E. Friedrich, *Pharmazie* **1977**, *32*, 730.

[6] B. Hladon, W. Bylka, M. Ellnain-Wojtaszek, L. Skrzypczak, P. Szafarek, A. Chodera, Z. Kowalewski, *Arzneim. Forschung* **1980**, *30*, 1847.

[7] J.B. Harborne, *The Flavonoids*, Chapman and Hall, London, 1994, p. 330.

[8] V. Bankova, R. Christov, G. Stoev, S. Popov, *J. Chromatogr.* **1992**, *607*, 150.

[9] H. Chi, A.K. Hsieh, C.L. Ng, H.K. Lee, S.F.Y. Li, *J. Chromatogr. A* **1994**, *680*, 593.

[10] V. Katalinic, *J. Chromatogr. A* **1997**, *775*, 359.

[11] P. Andrade, F. Ferreres, M.T. Amaral, *J. Liq. Chromatogr.* **1997**, *20*, 2281.

[12] V.S. Bankova, S.S. Popov, N.L. Marekov, *J. Chromatogr.* **1982**, *242*, 135.

[13] U. Seitz, P.J. Oefner, S. Nathakarnkitkool, K. Bonn, *Electrophoresis* **1992**, *13*, 35.

[14] T.K. McGhie, *J. Chromatogr.* **1993**, *634*, 107.

[15] F. Ferreres, M.A. Blazquez, M.I. Gil, F.A. Thomas-Barberan, *J. Chromatogr. A* **1994**, *669*, 268.

[16] P.G. Pietta, P.L. Mauri, L. Zini, C. Gardana, *J. Chromatogr. A* **1994**, *680*, 175.

[17] P. Pietta, P. Mauri, A. Bruno, L. Zini, *J. Chromatogr.* **1993**, *638*, 357.

[18] Y.M. Liu, S.J. Sheu, *Anal. Chim. Acta* **1994**, *288*, 221.

[19] H.R. Liang, H. Siren, M.L. Riekkola, P. Vuorela, H. Vuorela, R. Hiltunen, *J. Chromatogr. A* **1996**, *746*, 123.

[20] C. Bjergegaard, S. Michaelsen, K. Mortensen, H. Sorensen, *J. Chromatogr. A* **1993**, *652*, 477.

[21] P.G. Pietta, P.L. Mauri, A. Rava, G. Sabbatini, *J. Chromatogr.* **1991**, *549*, 367.

[22] H.R. Liang, H. Sirén, P. Jyske, M.L. Reikkola, P. Vuorela, H. Vuorela, R. Hiltunen, *J. Chromatogr. Sci.* **1997**, *35*, 117.

[23] P. Andrade, F. Ferreres, M.T. Amaral, *J. Liq. Chrom.* **1997**, *20*, 2281.

[24] E.P. Serjeant, B. Dempsey, *Ionisation constants of organic acids in aqueous solutions*, Pergamon, Oxford 1979,12th ed.

[25] S. Fujiwara, S. Honda, *Anal. Chem.* **1986**, *58*, 1811.

[26] M.J. Hilhorst, G.W. Somsen, G.J. de Jong, *J. Pharm. Biomed. Anal.* **1998**, *16*, 1251.

[27] H. Nishi, T. Fukuyama, M. Matsuo, S. Terabe, *J. Chromatogr.* **1990**, *513*, 279.

[28] H. Nishi, M. Matsuo, *J. Liq. Chrom.* **1991**, *14*, 973.