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On-line coupling of capillary isotachophoresis and capillary zone electrophoresis for the determination of flavonoids in methanolic extracts of *Hypericum perforatum* leaves or flowers

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Abstract

Five flavonoids (hyperoside, isoquercitrin, quercitrin, quercetin and rutin) were separated and determined in extracts of *Hypericum perforatum* leaves or flowers by capillary zone electrophoresis (CZE) with isotachophoretic (ITP) sample pre-treatment using on-line column coupling configuration. The background electrolyte (BGE) used in the CZE step was different from the leading and terminating ITP electrolytes but all the electrolytes contained 20% (v/v) of methanol. The optimal leading electrolyte was 10 m*M* HCl of pH*≈7.2 (adjusted with Tris) and the terminating electrolyte was 50 m*M* H_3BO_3 of pH*≈8.2 (adjusted with barium hydroxide). This operational system allowed to concentrate and pre-separate selectively the flavonoid fraction from other plant constituents before the introduction of the flavonoids into the CZE capillary. The BGE for the CZE step was 50 m*M* Tris buffer of pH*≈8.75 containing 25 m*M* *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid as co-ion and 55 m*M* H_3BO_3 as complex-forming agent. The ITP–CZE method with spectrophotometric detection at 254 nm was suitable for the quantitation of the flavonoids in real natural samples; kaempferol was used as internal standard. The limit of detection for quercetin-3-*O*-glycosides was 100 ng ml^{-1} and calibration curves were rectilinear in the range 1–10 μg ml^{-1} for most of the analytes. The RSD values ranged between 0.9 and 2.7% ($n=3$) when determining ≈0.07–1.2% of the individual flavonoids in dried medicinal plants.

Keywords: *Hypericum perforatum*; Plant materials; Isotachophoresis–capillary zone electrophoresis; Flavonoids; Quercetin glycosides; Glycosides

1. Introduction

Chromatographic techniques such as HPLC, GC and HPTLC have occupied the leading position in the area of phytopharmaceutical analysis in the last 20 years [1–4]. These methods that are routinely used in the pharmaceutical industry have also been implemented in leading world pharmacopoeias to ensure quality and safety of drugs of natural origin including medicinal plant extracts. Because of the rather complex character of plant materials, time-consuming sample pre-treatment involving solid-phase extraction (SPE) or liquid–liquid extraction is frequently required to remove the sample matrix

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prior to the chromatographic step [5–9]. SPE can be realised in SPE well plates, especially in routine analyses of a large series of samples. Only a few papers dealing with direct HPLC analysis of complex natural samples employing column switching have been published [10,11]. Valuable features of electromigration analytical techniques (CE, micellar electrokinetic chromatography and capillary electrochromatography), i.e. high separation efficiency and short time of analysis allow them to be applied for the assays of pharmaceutically and biomedically important compounds in plant materials and similar natural products [12,13]. On the other hand, compared to HPLC, these electrophoretic techniques generally exhibit much lower sensitivity, facility to sample overloading (that leads to the loss of separation efficiency) and less reproducible quantitative data. The limits of detection reached by electromigration methods can be substantially improved for example by sample stacking (30–100 times), by isotachophoretic sampling or by transient isotachophoresis (20–1000 times) [14–17] and by on-line SPE–CE coupling realised by a ligand bound directly to inner capillary surface or to silica beads (1000 or 50–7000 times, respectively) [18–22]. While on-line coupling of capillary isotachophoresis (ITP) and capillary zone electrophoresis (CZE) in a single column leads merely to the improvement of limits of detection, the column coupling arrangement (with an additional ITP column of relatively large inner diameter used as the CE column) may also improve the selectivity and efficiency of separation [23]. In this case, ITP plays the role of on-line sample pre-treatment enabling pre-concentration and separation of minor or trace components (analytes) of interest from samples with a complex matrix [24]. The same holds for on-line combination of SPE–CE [18–22]. These concepts lead to the improvement of the sample loading ability. Even though these on-line coupling techniques offer considerable improvement of CE performance, their application in routine analyses of complicated samples in biomedical, clinical and pharmaceutical practice is not yet widespread as might be expected. Instrumentation for on-line combination of ITP–CZE in column coupling arrangement is not usually available from leading manufacturers of CE equipment. The optimisation of separation conditions (selection of suitable buffers and proper timing of current switching in column coupling tandem) is more complicated in comparison to the conventional CZE separations carried out in a single capillary at constant voltage [25,26]. Realisation of on-line SPE–CE separations faces problems with finding suitable commercial instrumentation (laboratory-made equipment is usually employed) and with the stability of SPE column packings (modified silica particles) at the extremely low or high pH values of buffers employed. Reproducible regeneration of capillary and SPE material after analysis of biological samples may also be difficult. The present paper deals with the optimisation of electrophoretic separation of quercetin-3-*O*-glycosides occurring in extracts of *Hypericum perforatum* leaves and flowers using on-line isotachophoretic sample pre-treatment in the column coupling arrangement. *Hypericum perforatum* L. is a herbaceous perennial plant, belonging to the Hypericaceae family, common in Europe, Asia, Northern Africa and naturalized in the USA. *H. perforatum* contains a number of constituents with documented biological activity including chlorogenic acid, a broad range of flavonoids, naphthodianthrones and phloroglucinols. The dried flowers or aerial parts are used for the preparation of a drug called Hyperici Herba or St. John's Wort. Nowadays this drug is largely used as a natural antidepressant; hypericin and hypericin-like substances are considered to be the main active ingredients but it seems that flavonoid constituents could participate in the antidepressant activity of the extract too [27–29]. On the other hand, several recent reports indicate that hyperforins are responsible for the main activity of such extracts [30]. The biflavonoids, such as amentoflavon, might also contribute to the sedative effect of the drug because it was shown that amentoflavon has a strong affinity to the benzodiazepine binding site in γ-aminobutyric acid receptors [27,31]. This medical plant has a high reputation as an anti-inflammatory and healing agent and antiviral and anti-tumour activities have also been attributed to its extracts [27]. So far mostly HPLC methods for the assay of hypericin and pseudohypericin in *Hypericum* extract [32–36], in phytotherapeutic herbal and alcoholic beverages [37] or in pharmaceutical preparations [38,39] have been published. Hypericin and/or hyperforin were monitored in blood plasma by HPLC [40,41]. Several

papers dealing with HPLC analysis of all active ingredients in *Hypericum* extract [42–46] or in dietary supplements [47,48] were published. In these papers, photodiode array and mass spectrometric detection are often used to identify most of the compounds. A few HPLC papers focusing mainly on the flavonoid fraction have been published [32,49]. TLC–densitometry was used for the identification and determination of hypericins in extract [32,50] or for flavonoids fraction [51,52]. To our best knowledge, only two earlier papers were devoted to CE analysis of *Hypericum* extract; the authors [53] were dealing with non-aqueous capillary electrophoresis of hypericins and hyperforins in a system with reversed electroosmotic flow and in another paper a CE method for the determination of rutin has been devised [54].

2. Experimental

2.1. Chemicals

All chemicals used for the preparation of model mixtures and electrolyte solutions were of analytical grade. Hydrochloric acid, boric acid, tris(hydroxymethylamino)methane (Tris), barium hydroxide, picric acid, 1-nitroso-2-naphthol and methanol were obtained from Lachema (Brno, Czech Republic). β-Hydroxy-4-morpholinopropanesulfonic acid (MOPSO) and *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) were purchased from Fluka (Buchs, Switzerland). Chlorogenic acid, rutin, quercetin and 2-hydroxyethylcellulose (HEC) were obtained from Aldrich (Milwaukee, WI, USA). Isoquercitrin, quercitrin and hyperoside were purchased from Roth (Karlsruhe, Germany). Ultra pure water used throughout was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Plant materials

Hypericum leaves and flowers conforming to pharmacopoeial standards were obtained in pharmacy in the form of an antidepressant tea. The flowers of *Hypericum perforatum* were collected from plants grown in the Botanical Garden of the Faculty of Pharmacy, Hradec Králové in the middle of August 2001.

2.3. Standard solutions

Stock solutions containing 200 μg ml^{-1} of rutin, quercetin or chlorogenic acid were prepared by dissolving the compounds in methanol. Stock solutions containing 40 μg ml^{-1} of isoquercitrin, quercitrin or hyperoside standards were prepared in aqueous 20% (v/v) methanol. A stock solution of internal standard kaempferol (100 μg ml^{-1}) was prepared in methanol. The final model mixture **A** containing 10 μg ml^{-1} of each analyte in 20% (v/v) methanol was prepared from the stock solutions of the standards. Calibration solutions prepared by dilution of mixture **A** contained 10 μg ml^{-1} of kaempferol as internal standard.

2.4. Electrolyte solutions

The ITP and CZE electrolytes contained 20% (v/v) of methanol; 2-hydroxyethylcellulose (0.2%) was used as an additive to the leading (LE) and background (BGE) electrolyte solutions. The pH of the electrolytes was adjusted after the addition of methanol and therefore the pH values measured are apparent (pseudo) values denoted here as pH*. All the electrolytes were filtered through sintered glass filter No. 4 and degassed for 15 min in an ultrasonic bath.

2.5. Preparation of plant extracts

Flavonoids were extracted from 0.5 g of dried pulverized leaves and flowers or flowers of *H. perforatum* into 50 ml of methanol by sonication for 30 min. Thereafter the suspension was filtered through a dry paper filter, 1 ml of the supernatant was treated with 1 ml of methanol and with 2 ml of stock solution of kaempferol as internal standard and this solution was diluted to 20 ml with water before the ITP–CZE analysis.

2.6. Instrumentation

The ITP–CZE experiments were carried out using the EA 100 VILLA Labeco CS Isotachophoretic

analyser (Spišská Nová Ves, Slovakia) equipped with a column switching system. Pre-separation and analytical capillaries were made of fluorinated ethylene–propylene copolymer (FEP). The pre-separation capillary (9.0 cm×0.8 mm I.D.) was equipped with a conductivity detector positioned at a distance of 38 mm from the bifurcation point. The analytical capillary (16 cm×0.3 mm I.D.) involved conductivity as well as spectrophotometric (254 nm) detectors. Test solutions (30 μl) were injected using a sampling valve. The electrolyte chambers containing the LE and BGE were separated from the electrolytes in the capillaries by semi-permeable cellophane membranes. The temperature of the CZE capillary was maintained at 25 °C using a laboratory-made thermostat based on Peltier elements. Experimental data were acquired and processed by personal computer software purchased from VILLA. The pH (or pH*) was measured by PHM-220 (Radiometer, France) pH meter equipped with pHC2401-8 combined glass electrode calibrated with standard Radiometer buffers.

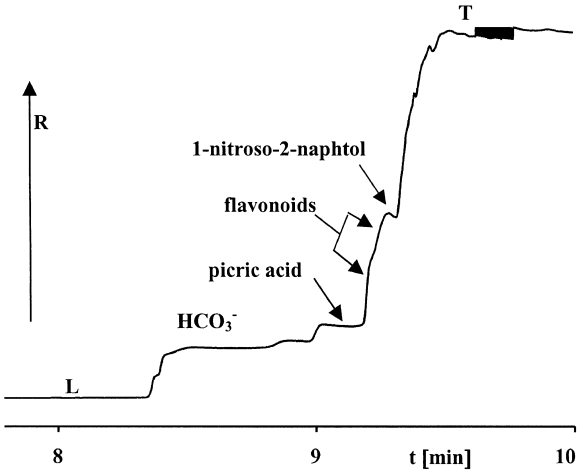

Fig. 1. Isotachophoreogram of a standard mixture of flavonoids and chlorogenic acid (≈10 μg ml^{-1}) with picric acid and 1-nitroso-2-naphthol as the colour markers. For optimal composition of ITP electrolytes, see Table 1.

3. Results and discussion

3.1. Selection of ITP electrolyte systems

The ITP operational electrolytes for sample pre-treatment were selected to ensure pre-concentration and separation of quercetin-3-*O*-glycosides from other phenolic constituents due to their selective electromigration. The main structural attribute common to these compounds is the presence of *ortho*-dihydroxyphenyl moieties known for their ability to form complexes with borate. The capability of hydroxy groups of sugars to form borate complexes may also enhance the efficiency of ITP pre-treatment. Therefore boric acid solution of pH* 8.2 (adjusted with barium hydroxide) was used as the terminating electrolyte (TE). Due to this complexation the flavonoids migrate at relatively low value of pH*≈8.0 whereas other constituents whose migration depends just on the ionisation of the phenolic groups cannot form sharp ITP zones. The leading electrolyte was hydrochloric acid containing Tris as the counter-ion (Table 1). Picric acid and 1-nitroso-2-naphthol were used as the colour markers which made it possible to use the response of ITP conductivity detector for exact timing of introduction of ITP zones into CZE separation capillary (Fig. 1). The driving current during the ITP step was initially 200 μA (for 10 min) and thereafter it was decreased to 100 μA and maintained at this value until the current switching from ITP to the CZE capillary. The transfer of the analytes into the CZE capillary took

Table 1
The operational system used in the ITP pre-separation step

	LE	TE
Solvent	Water–methanol (4:1)	Water–methanol (4:1)
Anion	10 m*M* Cl^-	50 m*M* H_3BO_3
Counter-ion	Tris	–
pH[a]	7.20	8.20 [adjusted by $Ba(OH)_2$]
Additive	0.2% (w/v) HEC	

[a] Pseudo pH value measured in 20% (v/v) aqueous methanol.

200 s at a current of 50 μA. Thereafter the TE present in the pre-separation capillary and in the upper TE chamber was replaced by the BGE, the current was increased to 200 μA and the separation continued in the CZE capillary (Fig. 2).

3.2. Selection of the BGE co-ions for the CZE step

The optimal background buffer for electrophoretic separations with ITP sampling should be found with respect to the value of co-ion effective mobility. Only in the case when the value of effective mobility of background co-ion matches well those of separated analytes good separation efficiency of the analytes (pre-concentrated during the ITP step) in the CZE stage can be obtained. Therefore the LE and TE used in the ITP step cannot serve as BGE which was confirmed by preliminary experiments. On the other hand, sulfonic acid derivatives such as MOPSO and TAPS seem to be optimal co-ions since their

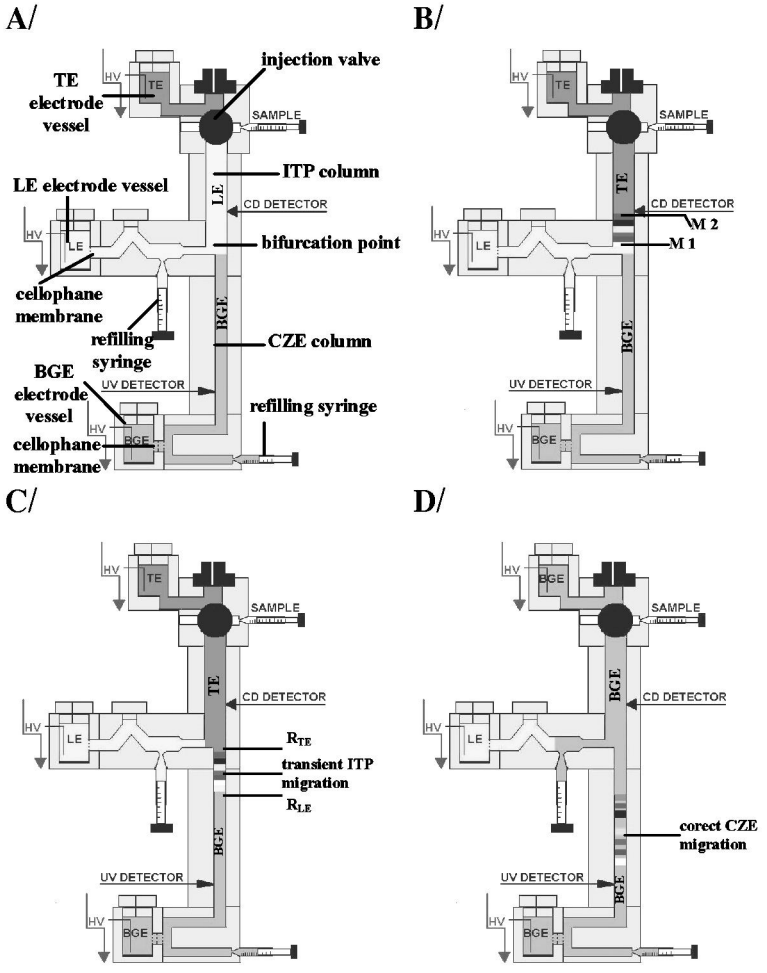

Fig. 2. Scheme for the separation unit for on-line combination of capillary isotachophoresis with capillary zone electrophoresis in column coupling arrangement. The running mode is the system of three different electrolytes (BGE–S–BGE). (A) Situation at the start of analysis. (B) The end of ITP pre-separation step when the analytes of interest are stacked between colour markers picric acid (M_1) and 1-nitroso-2-naphthol (M_2). (C) Introduction of ITP arranged stack into CZE column. (D) Electrophoretic migration of analytes after refilling the ITP column and TE electrode chamber with BGE electrolyte. For detailed description, buffer composition and the optimal time and current regime, see the text.

effective mobility values are close to those of quercetin-3-*O*-glycosides. This fact was verified by comparison of calculated effective mobility values of the analytes and BGE co-ions and experimentally from the ITP wave heights of MOPSO and TAPS obtained by ITP analysis of a mixture of the flavonoids spiked with the sulfonic acid derivatives cited above (cf. Section 3.6). The relation between the effective mobilities u of ions in the LE, TE and co-ions in BGE in our three-electrolyte system was: $u_T < u_{BGE} < u_L$. To realize the ITP–CZE on-line combination in such a system of three different electrolytes known as BGE–S–BGE [25,26] it was necessary to control and minimise the volume of LE or TE zones introduced into the CZE capillary together with the stack of analytes. When observing this requirement very good reproducibility of peak migration times and peak areas of the analytes was achieved. Since boric acid significantly influenced the selectivity of separation, the resulting BGE system represents a double buffer containing two anionic species—sulfonic acid as co-ion and the boric acid as complexing agent (Table 2).

3.3. Effect of pH

To estimate the effect of pH on the selectivity of quercetin-3-*O*-glycoside separation, the BGE buffers with 25 m*M* MOPSO as co-ion and 50 m*M* boric acid (the same concentration as in TE to guarantee complexation of phenolic and alcoholic hydroxy groups) with pH* values between 7.8 and 9 were tested (Fig. 3). The most difficult task was to separate the trio of quercitrin, isoquercitrin and hyperoside differing only in the type of sugar bound to the aglycon quercetin. The selectivity of separation of these compounds depends on the different

Table 2
The background electrolytes used in the CZE step

	BGE 1	BGE 2
Co-ion	MOPSO (25)	TAPS (25)
Counter-ion (m*M*)	Tris (50)	Tris (50)
Complexing agent (m*M*)	H_3BO_3 (55)	H_3BO_3 (55)
Additive (%)	HEC (0.2)	HEC (0.2)
Solvent (%)	Methanol (20)	Methanol (20)
pH[a] [adjusted with $Ba(OH)_2$]	8.3	8.75

[a] Pseudo pH value measured in 20% (v/v) aqueous methanol.

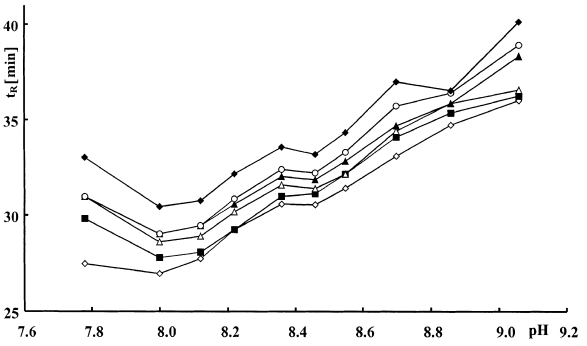

Fig. 3. Effect of pH* on the separation of flavonoids and chlorogenic acid. Concentration of boric acid 50 m*M*. Quercetin ◇, chlorogenic acid ■, quercitrin △, isoquercitrin ▲, hyperoside ○, rutin ♦. For the separation conditions, see the text.

ability of sugars to form complexes with boric acid at different pH. In the pH* range between 7.8 and 8.2 quercitrin is separated only partly from isoquercitrin that migrates together with hyperoside. At pH* 8.36, good resolution was observed for all six analytes. Further increase in pH* (8.46–8.9) did not lead to improvement of resolution; quercitrin and isoquercitrin migrated faster due to stronger sugar complexation. At pH* 8.55, quercitrin migrated together with chlorogenic acid and in the pH* range 8.7–8.9, poor separation of chlorogenic acid, quercitrin and isoquercitrin was obtained. In buffers with pH*≥9, no improvement of selectivity was reached and moreover the peak shape and symmetry deteriorated.

3.4. Effect of concentration of boric acid

The effect of 10–95 m*M* boric acid on the selectivity of separation in the background buffers of pH* 8.36 with 25 m*M* MOPSO as co-ion is shown in Fig. 4. Presumably at constant pH, the changes in effective mobilities of quercitrin, isoquercitrin and hyperoside take place due to the complexation of their sugar moieties. The optimum boric acid concentration ensuring the separation of all compounds down to the baseline was found to be 50 m*M*. Further increase in borate concentration to 60 m*M* significantly influenced the quercitrin and isoquercitrin mobilities; this resulted in incomplete separation of quercitrin from chlorogenic acid; in ≥70 m*M* borate, these two analytes migrate together.

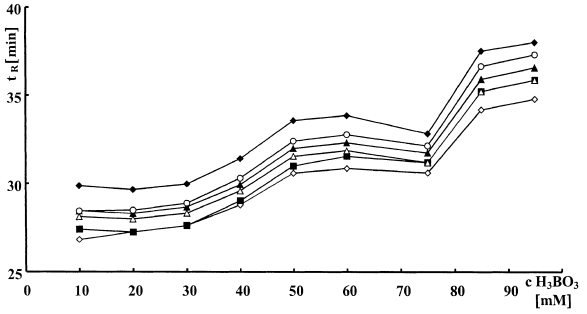

Fig. 4. Effect of the concentration of boric acid in the BGE on the migration behaviour of flavonoids and chlorogenic acid. pH*≈ 8.35; Quercetin ◇, chlorogenic acid ■, quercitrin △, isoquercitrin ▲, hyperoside ○, rutin ♦. For the other conditions, see the text.

At ≥85 m*M* borate, good resolution of isoquercitrin from hyperoside was achieved.

3.5. The effect of the addition of cyclodextrins

After further ''fine-tuning'' of the separation conditions, the buffer containing 25 m*M* MOPSO, 55 m*M* boric acid and 50 m*M* Tris adjusted with barium hydroxide to pH*≈8.30 was found as optimal BGE (Fig. 5A). In an attempt to attain additional improvement of the resolution of the analytes the effect of 5–20 m*M* α-, β-, γ- or carboxymethyl-β-cyclodextrin added into the BGE was tested. It was supposed that some of the borate complexes of flavonoids could interact selectively with cyclodextrins. All the cyclodextrins examined except for α-cyclodextrin exhibited non-selective interactions with all flavonoids tested; this reflected in the increase in migration times and in the deterioration of resolution. In the presence of 20 m*M* α-cyclodextrin only chlorogenic acid formed an inclusion complex; this reflected in its much slower migration so that the peak of chlorogenic acid was detected behind that of the slowest flavonoid rutin (Fig. 5B). Because of the higher buffer viscosity the time of analysis was prolonged and better resolution of analytes was reached. It must be noted that only higher concentration of α-cyclodextrin could influence the chlorogenic acid migration so significantly. With 5–10 m*M* α-cyclodextrin, no interactions with chlorogenic acid were observed while at 15 m*M* concentration, incomplete complex formation resulted in chlorogenic acid migrating together with rutin.

3.6. The effect of the background co-ion

The two optimal MOPSO-based systems (without and with α-cyclodextrin) mentioned above with different selectivity for chlorogenic acid were used for the analysis of real plant samples. In view of the fact that the resolution of peaks was not so good as with the mixture of standards and accurate quantitative determination of all flavonoids was not possible, another modification of the BGE was necessary. Another possibility how to improve the efficiency of separation was the change in the background co-ion; this approach is usually not so effective in conventional CZE as in electrophoretic separations with transient ITP migration (ITP–CZE in column cou-

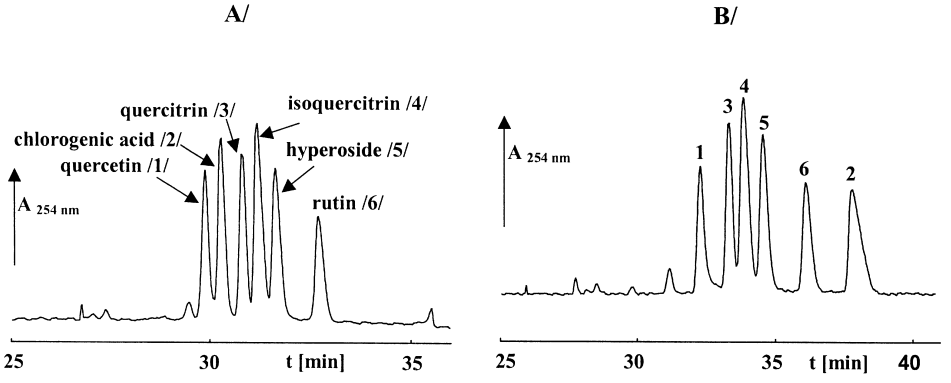

Fig. 5. The effect of α-cyclodextrin on the CZE separation. MOPSO as carrier anion in BGE 1 (Table 2); (A) BGE without α-cyclodextrin; (B) BGE with 20 m*M* α-cyclodextrin.

pling arrangement or ITP–CZE realised in a single capillary). The change in background co-ion can influence the duration of transient isotachophoretic migration in the separation electrophoretic column. When the time of ITP migration in this mode is shortened, the analytes start to migrate in electrophoretical regime earlier; hence the distances between CE peaks are enlarged and higher resolution can be reached. Comparison of the apparent effective mobility u^*_{eff} of MOPSO (pK_a=6.79; u_i=23.8×10^{-9} m^2 V^{-1} s^{-1}; u^*_{eff}=23.1×10^{-9} m^2 V^{-1} s^{-1} at pH* 8.3 in 20% aqueous methanol) with u^*_{eff} values of quercetin and rutin (12.98 and 10.84×10^{-9} m^2 V^{-1} s^{-1}, respectively) suggests that replacement of MOPSO by another co-ion with lower u^*_{eff} would be reasonable. The other choice was 25 m*M* TAPS (pK_a=8.30; u_i=25.0×10^{-9} m^2 V^{-1} s^{-1}; u^*_{eff}= 18.45×10^{-9} m^2 V^{-1} s^{-1} at pH* 8.75 in 20% aqueous methanol with 55 m*M* boric acid and 50 m*M* Tris) in the BGE. With this electrolyte system, excellent baseline resolution of all selected flavonoids was achieved not only for a standard mixture but the same results were obtained with plant extract too (Fig. 6). Nevertheless, the peaks of chlorogenic acid and isoquercitrin overlapped.

3.7. Determination of quercetin-3-O-glycosides in H. perforatum leaves and flowers

Despite the fact that the ITP–CZE analyser employed was not equipped with a sophisticated diode-array spectrophotometric detector, relatively reliable identification of the flavonoids in extracts was facilitated due to the application of two electrophoretic buffers with different co-ions and pH values (compliance of different migration times of analytes in either buffer) and also using the technique of addition of reference compounds leading to the increase in heights of the relevant peaks. The ITP–CZE carried out in the first MOPSO-based BGE of pH*≈ 8.3 showed that the chlorogenic acid does not occur in our plant material but quercitrin, isoquercitrin, hyperoside, rutin and their aglycon quercetin were identified in analysed samples using this buffer system. With respect to these findings confirming the absence of chlorogenic acid in the real samples the TAPS-based BGE that enabled excellent separation

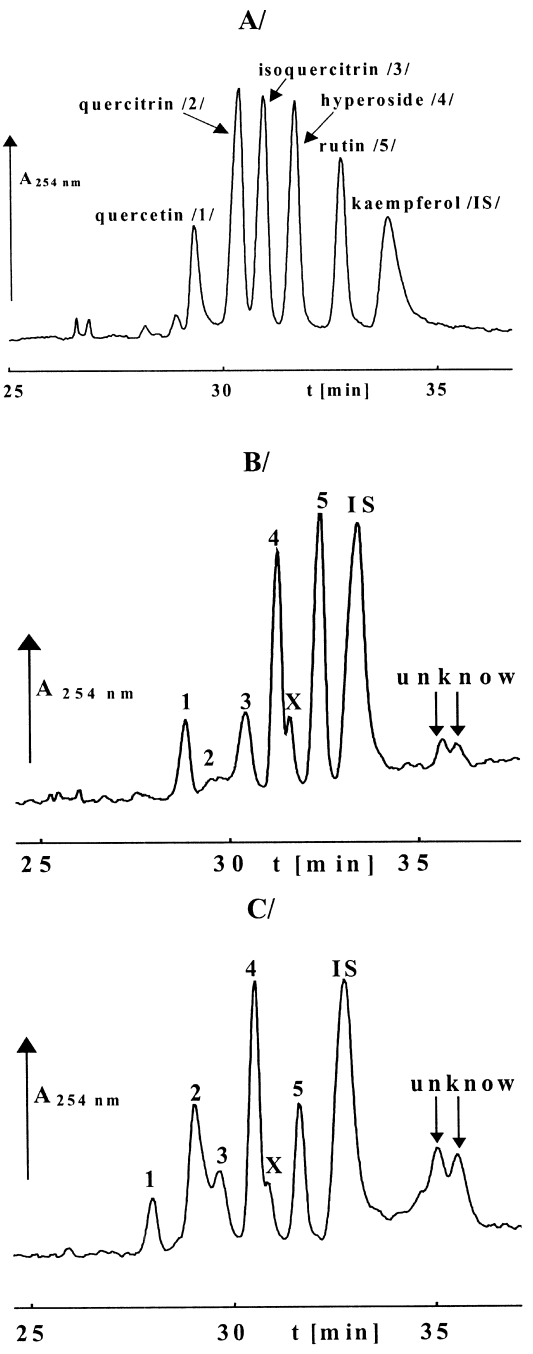

Fig. 6. ITP–CZE electrophoreograms of quercetin and quercetin-3-*O*-glycosides. Separation conditions: BGE No. 2 with TAPS as co-ion (Table 2); (A) standard mixture; (B) methanolic extract from *Hypericum* leaves and flowers; (C): *Hypericum* flowers.

of quercetin and its glycosides (Fig. 6B,C) could be used for assay of the flavonoids in extracts. To improve the linearity of calibration curves, kaempferol was used as the internal standard. The peak of this aglycon was detected closely behind the peak of rutin appearing as the last one on the electrophoreogram. The calibration curve was plotted for each analyte in the concentration range 1 −10 μg ml^{-1} (covered by seven experimental points, each measured in triplicate) except for quercetin where the range was 2 −10 μg ml^{-1}. Statistical evaluation of the calibration data, i.e. peak area ratio (analyte/internal standard) versus sample concentration (in μg ml^{-1}) confirmed very good linearity expressed by correlation coefficient values ≥0.9992 in each instance. The results of replicate analyses (n=6) of prepared mixture containing 5 μg ml^{-1} of each analyte showed RSD<2% (except for quercetin where the RSD was 7%) thus indicating good repeatability of the results. When comparing the linear range of calibration curves obtained by the present ITP–CZE method and previous HPLC methods [42,46,47] it can be noted that they are in the same concentration level (μg ml^{-1}) but the HPLC calibration ranges are wider (e.g. rutin 4.5–100 μg ml^{-1} [42]). All four quercetin-3-*O*-glycosides and their aglycon quercetin were quantified and the content of these compounds was expressed as percentage in the dry mass of plant material analysed. Intra-day assay precision was found to be ≤3% for all analytes except isoquercitrin. This value shows that the ITP–CZE method provides intra-day assay precision comparable to HPLC analyses published earlier [42,46,47]. The limit of detection (LOD; estimated as three times the signal-to-noise ratio [55]) for flavonoids was as low as 100 ng ml^{-1} (which is 50–200 times lower for rutin compared to conventional CE [54,56]) in spite of the fact that the analytical wavelength could not be optimised because of simplicity of the UV detector (only 254 nm could be used). The HPLC LOD values published for these compounds [11] show nearly the same values as the proposed ITP–CZE method; hence the pronounced influence of ITP concentrating effect on this important parameter was confirmed. The limits of quantitation (LOQs) evaluated according to Ref. [55] were 0.5 μg ml^{-1} for quercetin and 0.3 μg ml^{-1} for the rest of the flavonoids under study. The overall content of flavonoids in the *Hypericum* extracts found by the proposed ITP–CZE technique (Table 3) is in good accordance with literature data (2–4% of flavonoids) [27]. However, it is impossible to compare our results found for the individual flavonoids with the corresponding HPLC results published previously because the abundance and ratio of these flavonoids in analysed extract samples are variable depending on the origin of the parent plant, on the period of drug harvest and on the flower development stage.

4. Conclusion

This paper presents the first application of on-line combination of ITP–CZE in column coupling arrangement for qualitative and quantitative assay of quercetin and its glycosides in *H. perforatum* leaves and flowers. The main advantage of this hyphenated

Table 3
The determination of quercetin and its 3-*O*-glycosides in extract of *H. perforatum* leaves and flowers or flowers expressed as % content of compound in the dry mass

Analyte	*Hypericum* leaves and flowers[a] (%)	RSD (%) (n=3)	*Hypericum* flowers flowers[a] (%)	RSD (%) (n=3)
Quercetin	0.45	2.55	0.42	2.73
Quercitrin	0.07	2.21	0.71	0.82
Isoquercitrin	0.30	3.81	0.36	0.95
Hyperoside	0.66	1.74	0.94	2.22
Rutin	1.21	2.52	0.65	0.88
Total	2.69		3.08	

[a] Calculated on the dry mass.

electromigration technique consists of on-line sample pre-treatment including pre-separation and pre-concentration achieved by selective electromigration. The analytes can be concentrated from a relatively large volume of diluted extract sample (30 μl) by a factor of more than 1000 during this stage. The pre-concentration is fairly selective since only the compounds of interest have such acid–base and structural properties which enable them to be arranged in the ITP stack of sharp zones positioned between the zone of LE and TE. The subsequent on-line electrophoretic separation is characteristic of nearly baseline resolution of all compounds. Nevertheless the cleansing effect of the ITP pre-separation step cannot prevent ionised compounds of similar structure from entering the separation capillary and therefore they may be detected as interfering peaks when analysing natural samples. This situation is shown in Fig. 6B,C where an unknown peak X is not sufficiently resolved from that of hyperoside. In the electrophoreogram of *Hypericum* flowers extract, an unspecified interfering peak makes the resolution of quercitrin and isoquercitrin difficult. The reproducibility of quantitative analysis is satisfactory and the overall time of ITP–CZE analysis (35 min) compares well with the duration of such type of analyses performed by HPLC. The realisation of such hyphenated electromigration analysis is more economical in comparison to HPLC since the consumption of electrolytes is negligible and the use of organic solvents is practically avoided. The on-line ITP–CZE method for the determination of quercetin-3-*O*-glycosides in *H. perforatum* extracts described in this paper seems to be adequate for its application in routine analysis. The identification potency of the proposed method could be considerably improved by equipping the set-up with a diode-array UV detector.

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