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Isotachophoretic analysis of flavonoids and phenolcarboxylic acids of relevance to phytopharmaceutical industry

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ABSTRACT

Using capillary isotachophoresis, the rapid analysis of flavonoids and phenolcarboxylic acids was accomplished in the nanomole range. Optimum separations were achieved at pH 9.5 with a leading electrolyte containing 15 m*M* hydrochloric acid, 30% methanol and 0.2% hydroxypropylmethylcellulose. Under these conditions, the content of rutin could be determined in a methanolic extract of *Sambuci flos*, which is a common constituent of phytopharmaceutical products.

INTRODUCTION

Flavonoids are ubiquitous secondary plant metabolites. Their structures are based on 2-phenylbenzopyrone. The individual flavonoids vary in their degree of saturation and the pattern of substitution. In plants they exist mostly as mono- or diglycosides [1]. Flavonoids possess many pharmacological properties, of which antiphlogistic, spasmolytic, antiallergic, diuretic and anthelmintic activities are only a few [1]. In plants, flavonoids are often accompanied by phenolcarboxylic acids such as the analogues of cinnamic acid [2]. Because of the importance of flavonoids as remedies for inflammatory processes, their analysis is of considerable interest.

Existing methods commonly used for the analysis of flavonoids are high-performance liquid chromatography [3–7], thin-layer chromatography [8–11] and gas chromatography [12]. However, these analytical techniques tend to be time-consuming and laborious. Based on our experience with capillary isotachophoresis in the analysis of organic acids [13–17], its utility as a simple, rapid and economic alternative for the analysis of flavonoids and phenolcarboxylic acids in plant extracts was evaluated.

In the present study we investigated the influence of the pH and the percentage

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of methanol in the leading electrolyte on the resolution of flavonoids and phenolcarboxylic acids for their analysis in plant extracts.

EXPERIMENTAL

Sample pretreatment

All reference standard solutions were prepared from chromatographic- or analytical-reagent-grade chemicals (Sigma, St. Louis, MO, USA; Carl Roth, Karlsruhe, Germany) in methanol–water (80:20, v/v) at a concentration of 1 mg/ml. Flavonoids were extracted from 1 g of dried, pulverized flowers of *Sambucus nigra* (Caprifoliaceae) by means of 10 ml of methanol (Art. 6009, Merck, Darmstadt, Germany) for 5 min in a water bath kept at a temperature of 60°C. Subsequently, the sediment was filtered off (Art. 311642, Schleicher & Schuell, Dassel, Germany).

Isotachophoretic conditions

Isotachophoretic analyses were carried out on a LKB (Bromma, Sweden) Model 2127 tachophor equipped with a 250-mm PTFE capillary of 0.5 mm I.D.

The leading electrolyte was 15 m*M* hydrochloric acid (Art. 9057, Merck) in either water or mixtures of water and methanol (Art. 6009, Merck). The pH was adjusted to 8.5–9.5 by the addition of 2-amino-2-methyl-1,3-propanediol (Ammediol, A-2676, Sigma). The terminating electrolyte was 10 m*M* glycine (161-0718, Bio-Rad, Richmond, CA, USA) in methanol–water (30:70, v/v) adjusted to pH 10.6 by adding barium hydroxide (Art. 1737, Merck). Barium hydroxide precipitates the bicarbonate ions, which originate from dissolved carbon dioxide in alkaline media. Hydroxypropylmethylcellulose (HPMC, viscosity of 2% solution 4.000 centipoises, H7509, Sigma) was added to both the leading and the terminating electrolyte to reduce electroendosmosis.

The samples were injected through the inlet membrane into the leading electrolyte by means of 10-μl Hamilton syringes (Hamilton Bonaduz, Bonaduz, Switzerland). Usually separations were started at a current of 210 μA, which was gradually reduced to 60 μA shortly before the separated anions could be detected by means of their conductivity and differential conductivity. Analyses were carried out at a temperature of 20°C and took about 30 min.

RESULTS AND DISCUSSION

The phenolic nature of flavonoids indicates that the addition of organic solvents to the electrolytes may have a significant impact on their separation. Although flavonoids are soluble in alkaline media [1], their solubility is increased considerably by dissolving them in methanol, which belongs, like water, to the group of amphiprotic solvents with relatively high dielectric constants. Moreover, the acid–base characters of water and methanol do not differ much [18]. As long as the percentage of methanol does not exceed 30%, standard buffer solutions in water can be used to calibrate the pH meter in order to measure the pH of a methanol–water mixture correctly [19]. No corrections of the observed pH have to be made.

Using aqueous alkaline buffers, the investigated compounds, namely caffeic acid, luteolin, ferulic acid, chlorogenic acid, kaempferol, isoquercitrin, hyperosid and

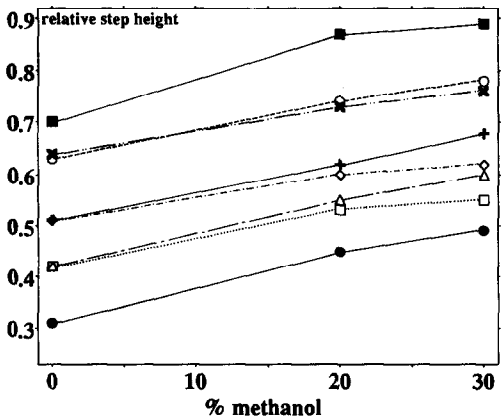

Fig. 1. Impact of the content of methanol in the running buffers on relative step heights (glycine = 1). Leading electrolyte: 15 m*M* hydrochloric acid–ammediol–0.2% HPMC, pH 9.5. Terminating electrolyte: 10 m*M* glycine–barium hydroxide–0.2% HPMC, pH 10.6. (●) Caffeic acid; (□) luteolin; (△) ferulic acid; (◇) chlorogenic acid; (+) kaempferol; (×) isoquercitin; (○) hyperosid; (■) rutin.

rutin, were resolved poorly. Owing to the change in solvation, resolution increased significantly upon addition of 20% methanol to the electrolytes. As Fig. 1 illustrates, optimum separability was obtained at a content of 30% methanol. This even allowed the separation of kaempferol and chlorogenic acid, which could not be resolved under aqueous conditions.

The parameter which primarily influences the effective mobilities of the analytes is the degree of dissociation. Because of their phenolic nature, an alkaline buffer system was chosen in order to ensure an adequate degree of dissociation of the flavonoids of interest. Fig. 2 shows the isotachophoretic analysis of a mixture of flavonoids and phenolcarboxylic acids commonly found in plant extracts at three different pH values of the leading electrolyte. The mixture contained caffeic acid, luteolin, chlorogenic acid, kaempferol, isoquercitrin and rutin. From the steepness of the steps in the conductivity signal it is evident that the highest resolution was obtained at a pH of 9.5. In addition, the third isotachopherogram indicates that the successful separation of flavonoids and phenolcarboxylic acids into individual zones is based on differences in negative charge and molecular weight. Compounds with lower molecular weight and larger numbers of free hydroxyls generally had greater mobility.

Fig. 3 shows the relative step heights of the investigated compounds in relation to glycine at pH 8.5, 9.0 and 9.5. The relative step heights of all compounds increased gradually with increasing pH of the leading electrolyte. However, as the change in mobility was not uniform, flavonoids such as kaempferol and isoquercitrin, which could not be separated at lower pH values, were resolved at a pH of 9.5. These results are contrary to those reported by Hiraoka *et al.* [20], who observed optimum resolution under neutral conditions.

By inhibiting electrode reactions and suppressing electroendosmosis, which is highest at high pH, surface-active compounds have a significant impact on boundary sharpness in isotachophoresis. While the investigated flavonoids were resolved rather

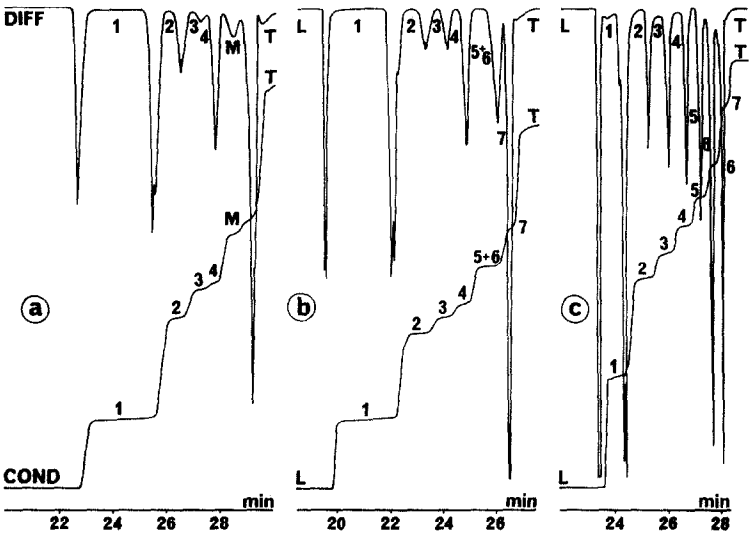

Fig. 2. Isotachopherograms of a standard solution of flavonoids and phenolcarboxylic acids at pH 8.5 (a), 9.0 (b) and 9.5 (c). Leading electrolyte (L): 15 m*M* hydrochloric acid–ammediol–0.2% HPMC in methanol–water (30:70, v/v). Terminating electrolyte (T): 10 m*M* glycine–barium hydroxide–0.2% HPMC in methanol–water (30:70, v/v), pH 10.6. Detection: conductivity (COND) and differential conductivity (DIFF). Current: 60 μA. Chart speed: 10 mm/min. Injection volumes: 2 μl. Zone identification: 1 = bicarbonate ions; 2 = caffeic acid; 3 = luteolin, 4 = chlorogenic acid; 5 = kaempferol; 6 = isoquercitin; 7 = rutin; M = mixed zones of kaempferol, isoquercitrin and rutin.

poorly when only 0.1% HPMC was added to the electrolytes, 0.2% HPMC was found to suppress electroosmotic flow efficiently and to ensure a constantly high resolution. Moreover, the increase in bulk viscosity might have also contributed to the enhanced boundary sharpness by reducing diffusion and convection effects.

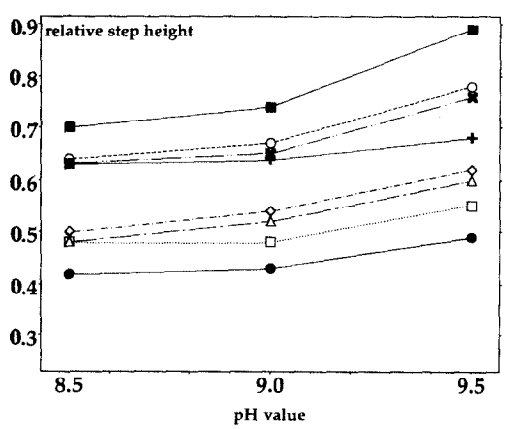

Fig. 3. Impact of the pH of the leading electrolyte on relative step heights (glycine = 1). Leading electrolyte: 15 m*M* hydrochloric acid–ammediol–0.2% HPMC in methanol–water (30:70, v/v). Terminating electrolyte: 10 m*M* glycine–barium hydroxide–0.2% HPMC in methanol–water (30:70, v/v), pH 10.6. Symbols as in Fig. 1.

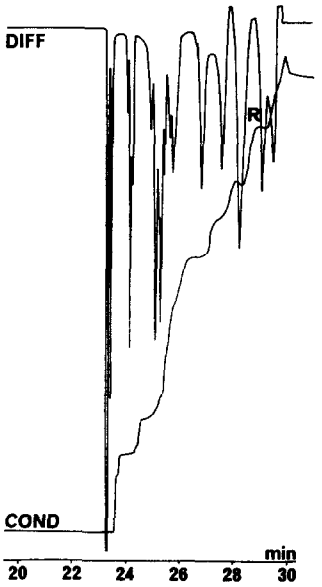

Fig. 4. Isotachophoretic analysis of a methanolic extract of *Sambuci flos*. Leading electrolyte: 15 m*M* hydrochloric acid–ammediol–0.2% HPMC in methanol–water (30:70, v/v), pH 9.5. Terminating electrolyte: 10 m*M* glycine–barium hydroxide–0.2% HPMC in methanol–water (30:70, v/v), pH 10.6. Detection: conductivity (COND) and differential conductivity (DIFF). Current: 60 μA. Chart speed: 10 mm/min. Injection volume: 3 μl. Zone identification: R = rutin.

Fig. 4 shows the presence of rutin, which is the flavonoid of greatest interest to phytopharmaceutical industry, in a methanolic extract of *Sambuci flos*. Its identity was confirmed by the injection of an additional small amount of a standard solution, which resulted in an increase in the length of the respective zone.

Quantitative information was obtained by measuring the zone length. The calibration curve for rutin revealed a detection limit of 3.8 nmol and linearity over its biological concentration range. In least-square linear regression analysis, the representative equation of the line and the regression coefficient were $y = 50.286x + 2.667$ and $r^2 = 0.994$, respectively.

By allowing the rapid and reproducible determination of flavonoids in plant extracts, the developed isotachophoretic method may serve as a valuable tool in assessing quality in the phytopharmaceutical industry.

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