Flavonoid Separation by Capillary Electrophoresis. Effect of Temperature and pH.

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Key Words

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pH effects

Summary

The use of capillary electrophoresis for the analysis of selected flavonols present in fruit juices and wines (kaempferol-3- rutinoside, rutin, avicularin, quercitrin, isoquercitrin, isorhamnetin, kaempferol and quercetin) was explored, and the effect of pH and temperature on the separation studied. The method had good reproducibility and analyses were carried out in less than 10 minutes.

Introduction

Flavonoids are a large group of naturally occurring substances widely distributed in vegetables, fruits and medicinal plants, and a great diversity of chemicals structures has been encountered. They have the basic skeleton of 2-phenylbenzopyrone, and differ in their degree of saturation and position of hydroxyl, methoxyl and sugar residues. Among the flavonoids, flavonols are one of the most important groups. They are the flavonoids with a higher degree of oxidation on the heterocycle. They were originally found in plants as conjugated glycosides or in the free state (aglycones), though from the literature it is not always clear whether or not aglycones may have been released during the extraction procedure [1]. We can find these substances not only in the original plant or fruit but also in the products derived from them, such as fruit juice and wine [2–4].

Analysis has mainly been by thin-layer chromatography (TLC) [5, 6] and reversed-phase high-performance liquid chromatography (RP-HPLC) [7–12]. The analysis of these compounds by HPLC is carried out on C-18 columns, using as mobile phase mixtures of water and organic solvents such as methanol, acetonitrile or tetrahydrofuran, with a small amount (1–5 %) of an acid (formic, acetic or phosphoric). The pH interval recommended is 2–7. Elution can be achieved in isocratic or gradient mode.

Recently, capillary electrophoresis (CE) has been proposed as a complementary technique [13, 14]. In this paper, the use of CE for the analysis of selected flavonols present in fruit juices and wines is explored, and the effect of pH and temperature on the separation studied. These compounds can be analyzed easily and rapidly by high-performance CE.

Experimental

Chemicals

Standard flavonols, including one kaempferol glycoside (kaempferol-3-rutinoside), four quercetin glycosides (quercetin-3-rutinoside -rutin-, quercetin-3-arabinoside -avicularin-, quercetin-3-rhamnoside -quercitrin- and quercetin-3-glucoside -isoquercitrin-) and three flavonol aglycones (isorhamnetin, kaempferol and quercetin) were from Extrasynthese (Genay, France). Boric acid and sodium hydroxyde were from Fluka (Buchs, Switzerland). Methanol was HPLC grade, and water Milli-Q^R, from Millipore (Massachusetts, USA).

The sample mixtures were prepared dissolving the above standards in methanol at a concentration of 1 mg ml^{-1}.

Apparatus and Conditions

High-performance CE was carried out using a Beckman (California, USA) P/ACE System 2050 equipped with a standard cartridge containing a 57 cm length (50 cm to detector) × 50 μm i.d. capillary. The detector was set at 280 nm. Sample injection was by pressure (0.5 psi) for 5 s. The temperature was maintained at 25, 30, 35 or 40 °C and the applied voltage was adjusted to 20 KV. Capillary conditioning was carried out by washing first with 0.1 M NaOH for 2 min, and then with water for 1 min. Finally, it was equilibrated by running buffer for

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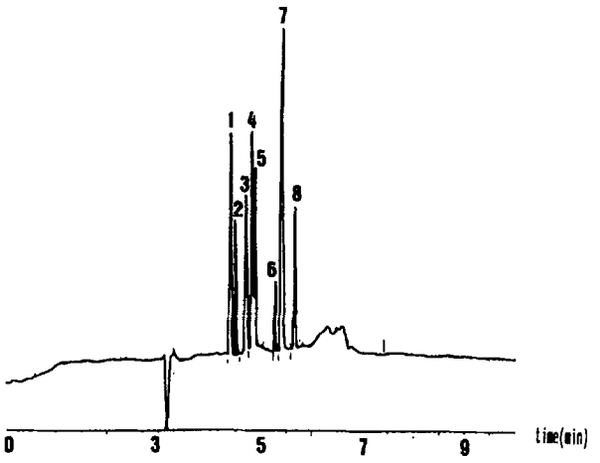

Figure 1
Electrophoregram of selected flavonols at pH 9.6, T = 25 °C and V = 20 KV. **1** = kaempferol-3-rutinoside, **2** = rutin, **3** = avicularin, **4** = quercitrin, **5** = isoquercitrin, **6** = isorhamnetin, **7** = kaempferol, **8** = quercetin.

2 min. Running buffers: 0.1 M boric acid, adjusting pH to 9, 9.5, 9.6, 10 and 11 by the addition of 1 M NaOH.

Results and Discussion

A good baseline separation of eight different flavonols is achieved in less than 10 min as illustrated in Figure 1, where the electrophoregram obtained with buffer pH 9.6, T = 25 °C and V = 20 KV can be seen. At the selected pH, the flavonols are negatively charged and would migrate away from the detector towards the anode. However, due to the large electroosmotic flow in the system, flavonols are propelled together with the bulk solution towards the cathode, but at a much lower rate. Those with highest molecular masses are detected first, since they are less able to migrate upstream. This is in accordance with the observation by Seitz et al. (1992) [13]. Therefore, all the glycosides are eluted before the aglycones. In the case of aglycones, the lowest migration time corresponds to isorhamnetin followed by kaempferol and quercetin. Among the glycosides analyzed, kaempferol glycoside is eluted before those of quercetin, and the migration time of quercetin derivatives increases as the molecular mass of the linked carbohydrate decreases.

Effect of Temperature

The electrophoretic mobility and the electroosmotic flow depend on viscosity. Viscosity is a function of temperature. As the temperature increases, the viscosity decreases; thus, the electrophoretic mobility increases as well. Figure 2 shows the effect of temperature on the electrophoretic mobility of flavonols at pH = 10 and 20 KV. Calculation of electrophoretic mobility (10^{-4} $cm^2V^{-1}s^{-1}$) was based on the migration time of the solute:

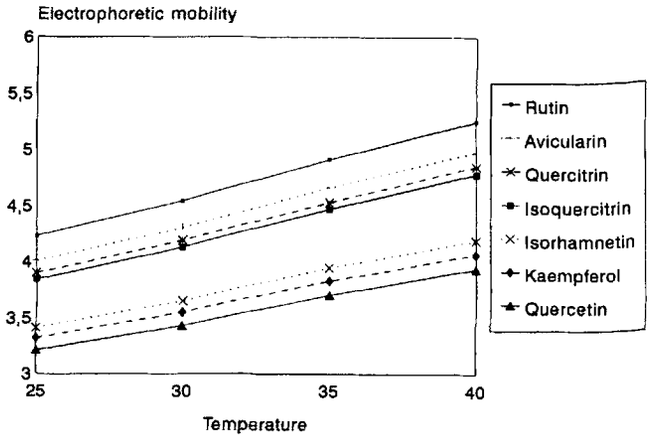

Figure 2
Effect of temperature on electrophoretic mobility of flavonols at pH 10 and 20 KV.

$$\mu_e = L_d \times L_t / V \times t_m$$

where L_t is total capillary length (cm), L_d capillary length to detector (cm), V applied voltage (V) and t_m solute migration time (s)

The migration time increases with temperature, and therefore the electrophoretic mobility increases. This increase is identical for all the flavonols studied. But, even though the effect of temperature on electrophoretic mobility is clear, there is no influence on resolution, and therefore it is not necessary to increase the temperature over 25 °C.

Effect of pH

The effect of pH on electroosmotic flow is substantial, and changes in peak resolution were induced by modifying the pH of the electrolyte. The pH range 9–11 was chosen to exploit better the influence of electroosmotic flow on electroosmotic mobility. Figure 3 shows the effect of pH on the electrophoretic mobility of flavonols at T = 25 °C and 20 KV. At pH 9 and 10 kaempferol-3-rutinoside and rutin have identical migration times. At pH 11, the procedure provided a somewhat poorer separation of flavonols, probably due to their complexation by borate [14]. The best overall separation for mixture components was obtained at pH 9.5 and 9.6. According to Pietta et al. (1994) [14], in this pH interval the electrophoretic behaviour is mainly influenced by the type of sugar linked to the aglycone.

Conclusions

Capillary electrophoresis, due to its high sensitivity and reproducibility, as well as its short run-times, is a good method for routine analysis of flavonoids. The effect of temperature in the electrophoretic mobility is identical for all flavonols studied, whereas the effect of pH depends on the linked carbohydrate.

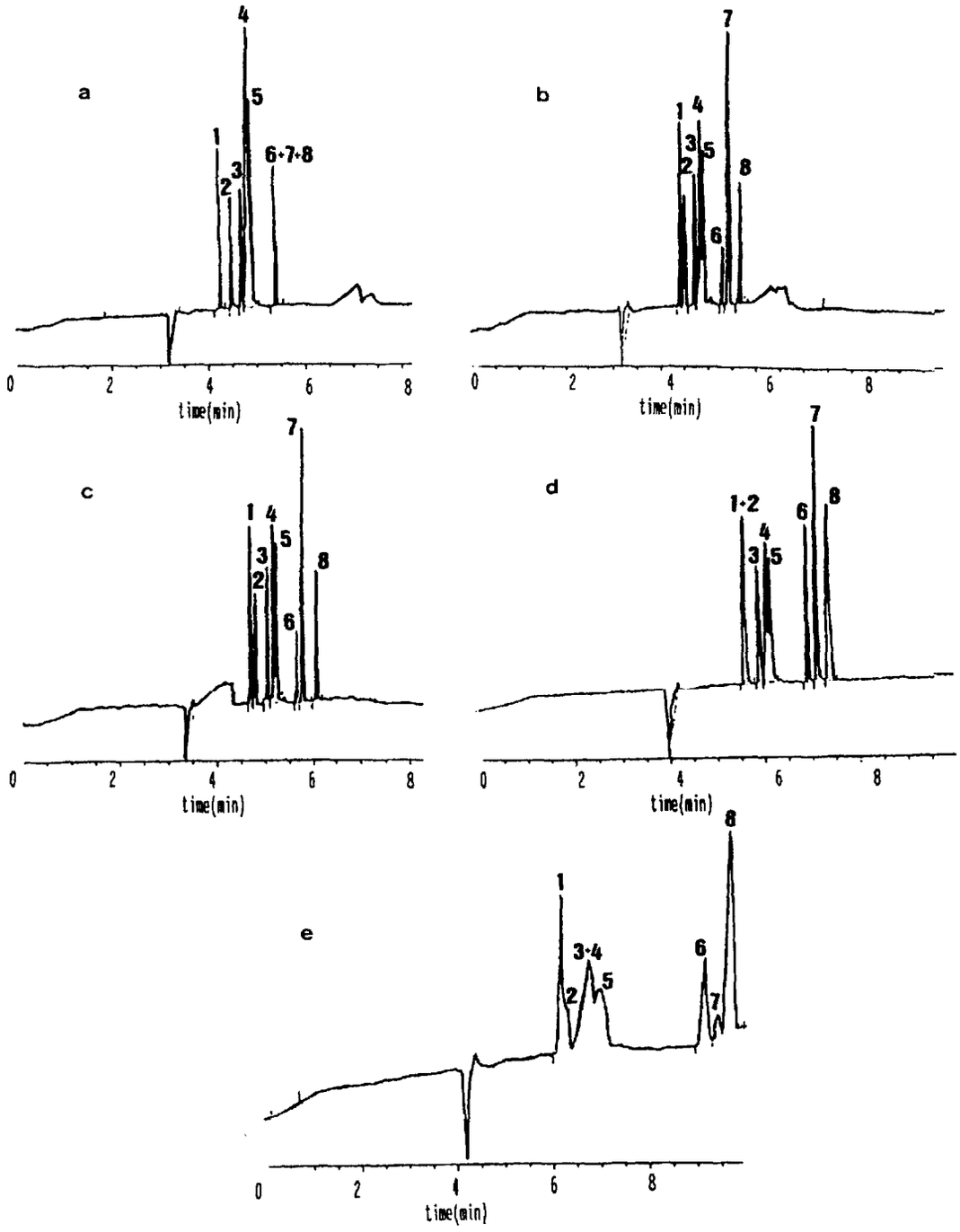

Figure 3
Electrophoregrams at different pH. **a** = 9; **b** = 9.5; **c** = 9.6; **d** = 10; **e** = 11.

Comparing the method used and the results obtained with those described in the literature for HPLC, CE presents certain advantages:

CE allows more analyses in the same time, since analysis time is shorter than 10 min, while 30 [3, 9, 10] or more minutes [8, 11, 12] are necessary for HPLC, so CE is faster.

CE does not require solvent gradients, mixtures of solvents, nor long column equilibration, necessary in analysis of these compounds by HPLC, so CE is simpler.

CE does not consume organic solvent (methanol, acetonitrile, THF, etc.) for elution, and in addition, the capillaries for CE are much less expensive than columns for HPLC, so CE is more economic.

HPLC presents other advantages. The use of elution gradients and organic solvents have a very direct influence in the separation of these compounds and in their resolution, and permits greater versatility, so it has wider application in separation of complicated natural mixtures.

References

[1] *J. B. Harborne,* "The flavonoids. Advances in research since 1980", Champman and Hall, London, 1988, p. 233.

[2] *B. Fernández de Simón, T. Hernández, C. Gómez-Cordovés, I. Estrella,* Abstracts of XVth International Conference Groupe Polyphenol,. Strasbourg, 1990, p. 91.

[3] *B. Fernández de Simón, J. Pérez-Ilzarbe, T. Hernández, C. Gómez-Cordovés, I. Estrella,* J. Agric. Food Chem. **40**, 1531 (1992).

[4] *I. Estrella, E. Alonso, E. Revilla,* Z. Lebensm. Unters Forch. **184**, 27 (1987).

[5] *K. R. Markham,* "Techniques of flavonoids identification", J. E. Treherne, P. H. Rubery, Academic Press, London, 1982.

[6] *E. Conde, E. Cadahía, M. C. García-Vallejo,* Chromatographia **33**, 418 (1992).

[7] *L. W. Wulf, C. W. Nagel,* J. Food Sci. **45**, 479 (1980).

[8] *V. Cheynier, J. Rigaud,* Am. J. Enol. Vitic. **37**, 248 (1986).

[9] *B. Fernández de Simón,* "Contribución al estudio de la maduración del fruto de *Vitis vinifera:* evolución de compuestos fenólicos C_6-C_1, C_6-C_3 y C_6-C_3-C_6", Universidad Complutense de Madrid, Madrid, 1990.

[10] *R. G. Bailey, I. McDowell, H. E. Nursten,* J. Sci. Food Agric. **52**, 509 (1990).

[11] *Y. S. Velioglu, G. Mazza,* J. Agric. Food Chem. **39**, 463 (1991).

[12] *J. Pérez-Ilzarbe, T. Hernández, I. Estrella,* Z. Lebensm. Unters Forsch. **192**, 551 (1991).

[13] *U. Seitz, P. J. Oefner, S. Nathakarnkitkool, M. Popp, G. K. Bonn,* Electrophoresis **13**, 35 (1994).

[14] *P. G. Pietta, P. L. Mauri, L. Zini, C. Gardana,* J. Chromatog. **680**,175 (1994).

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