Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 106 (2008) 415–420
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of potentially anti-carcinogenic flavonoids in wines by micellar electrokinetic chromatography

Ying Sun [a], Ning Fang [a], David D.Y. Chen [a], Kingsley K. Donkor [b,*]

[a] Department of Chemistry, The University of British Columbia, Vancouver, BC, Canada
[b] Department of Chemistry, Thompson Rivers University, Kamloops, BC, Canada

Received 9 December 2006; received in revised form 10 May 2007; accepted 29 May 2007

Abstract

A micellar electrokinetic chromatography (MEKC) is developed for the determination of potentially anti-carcinogenic flavonoids in various types of wines. The factors affecting the separation and detection, including the concentration and pH of the running buffer, the injection time, the sodium dodecyl sulphate (SDS) concentration, and the wavelength of UV absorption monitored were investigated to find the optimum conditions. Six potentially anti-carcinogenic flavonoids were separated within 16 min in a borate buffer containing SDS at pH 9.0. The detection limits for the six analytes were in the range of $1.48 \times 10^{-2} - 2.31 \times 10^{-2}$ μg mL^{-1}. The method was successfully used in the analysis of wines with a relatively simple extraction procedure.

Keywords: Flavonoids; Micellar electrokinetic chromatography; Wine; Capillary electrophoresis

1. Introduction

Flavonoids are a large group of phenolic compounds and constitute one of the largest groups of secondary metabolites in plants (De Rijke et al., 2006). The basic structure of flavonoids contains a phenolic ring with a 2-phenylbenzopyrone (Suntornsuk, 2002). Their derivatives differ in the substituents, the number and position of hydroxyl and methoxy groups, and the type and position of sugar moieties in the molecules (Michael, Hertog, Hollman, & Dini, 1992). Frequently, one or more of the hydroxyl groups are methylated, acetylated, prenylated or sulphated (De Rijke et al., 2006). Flavonoids exhibit important health benefits and pharmacological activities, such as anti-inflammatory, anti-allergy, anti-viral (Miean & Mohamed, 2001), anti-cancer (Bayard, Chamorro, Motta, & Hollenberg, 2007; Mak, Leung, Tang, Harwood, & Ho, 2006), anti-oxidant (Furusawa et al., 2005; Georgetti, Casagrande, Di Mambro, Azzolini, & Maria, 2003; Proestos, Boziaris, Nychas, & Komaitis, 2006; Yang, Kotani, Arai, & Kusu, 2001) and anti-microbial (Ielpo et al., 2000; Proestos et al., 2006). Other proven important properties of flavonoids include metal ion-chelation, enzyme inhibition, anti-proliferation, regulation of cell signaling and gene expression (Havsteen, 2002; Middleton, Kandaswami, & Theoharides, 2000). Flavonoids are also associated with a low incidence of osteoporosis and menopausal vasomotor symptoms such as hot flashes and night sweats (Powles, 2004). Auxins are hormones which are a critical determinant in controlling plant growth, and flavonoids have been found to act as auxin transport inhibitors (Brown et al., 2001). In addition, flavonoidic constituents are responsible for its astringency, colour, bitterness (Wang & Huang, 2004) and general organoleptic characteristics of wine. As a consequence, research interest in flavonoids has intensified due to its numerous health benefits and its relationship to the benefits of wine consuming in human diet. Analytical methods for flavonoids include HPLC (Bilbao, Andres-Lacueva, Jauregui, & Lamuela-Raventos, 2007; Da Queija, Queiros, & Rodrigues, 2001; Fang, Li, Pan, & Huang, 2007; Rehova, Skerikova, & Jandera, 2004; Wang & Huang, 2004), thin-layer

* Corresponding author.
E-mail address: kdonkor@tru.ca (K.K. Donkor).

0308-8146/$ - see front matter
doi:10.1016/j.foodchem.2007.05.094

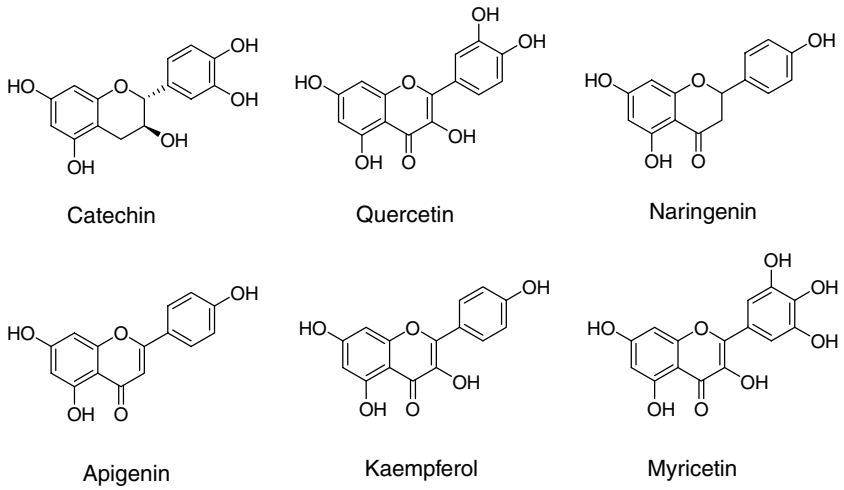

Fig. 1. The chemical structures of the investigated flavonoids.

chromatography (Soczewinski, Hawryl, & Hawryl, 2001), and gas chromatography–mass spectrometry (Fiamegos, Nanos, Vervoort, & Stalikas, 2004). However, in recent years, the popularity of capillary electrophoresis (CE) has increased dramatically since it offers several advantages, including excellent separation efficiency for complex samples, rapid analysis, minimum use of samples and organic solvents, much simpler and robust instrumentation, as well as various separation modes suitable for widely different analytes (Molnar-Perl & Fuzfai, 2005; Weinberger, 2000). MEKC, or surfactant mediated CE, offers superb selectivity in the analysis of complex substances (Dadakova, Prochazkova, & Krizek, 2001; Rodriguez-Delgado, Perez, Corbella, Gonzalez, & Montelongo, 2000; Tonin, Jager, Micke, Farah, & Tavares, 2005). Flavonoids in plants are complex and usually appear as mixtures, often with varied amount and quality (Wang & Huang, 2004). Several flavonoids have been determined by CE in different plant matrices (Suntornsuk, 2002; Volpi, 2004; Wang & Huang, 2004; Wu, Guan, & Ye, 2007). However, not that many analyses for flavonoids in wines have been carried out using capillary electrophoresis. Therefore, the general objective of this work is to characterize flavonoids by MEKC. The information obtained can be used to fingerprint wines and thus determine the botanical and geographical origin of the grapes used to make these wines. In an attempt to achieve this goal, we have focused on a selected number of flavonoids in wines that might have anti-carcinogenic effects (Da Queija et al., 2001). The structures of these flavonoids are shown in Fig. 1.

2. Experimental

2.1. Standards and reagents

Flavonoid standards, i.e., catechin, naringenin, quercetin, apigenin, kaempferol and myricetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The stock standard solutions of 200 mg L^{-1} of each analyte were prepared by dissolving the appropriate mass of the flavonoid in 20 mL of 1-propanol and then diluted with doubly deionized water to 100 mL. Resorcinol, sodium dodecyl sulphate (SDS) and sodium tetraborate (Borax) were also obtained from Sigma Chemical Co. (St. Louis, MO). 1-propanol was purchased from Fischer Scientific, Nepean, ON, Canada. All reagents were analytical-reagent grade and used without further purification. The background electrolyte (BGE) was prepared by dissolving the appropriate amounts of SDS and sodium tetraborate in doubly deionized water to obtain the final concentration. The samples and running buffers contained 20% 1-propanol. The pH of the running buffer was adjusted with either 1.0 M NaOH (BDH Chemicals, Toronto, ON, Canada) or 2.0 M HCl (Fischer Scientific, Nepean, ON, Canada) within a range of 8.0–10.0. All solutions were filtered through 0.22 μm sterile, Nylon filters prior to use for the CE experiments.

2.2. Instrumentation and electrophoretic procedure

All CE experiments were performed using a Beckman P/ACE MDQ capillary electrophoresis instrument (Beckman Coulter Inc., Fullerton, CA) equipped with a UV absorbance detector. Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) with inner diameters of 75 μm and total lengths of 60 cm (52 cm to the detector) were used. New capillaries were first rinsed with 1.0 M NaOH (30 min, 20 psi), followed by methanol for 20 min, 0.1 M NaOH for 20 min, deionized water for 10 min, and finally the BGE for 30 min, before being used. Sample injections were done by hydrodynamic pressure at 0.5 psi (3447 Pa). An injection time of 5 s was used for all analyses. UV absorption was monitored at 214 nm. The separation voltage was 15 kV at a constant temperature of 25 °C. The capillary was conditioned every day prior to use, with 0.1 M NaOH for 30 min followed by deionized water for 10 min, and finally the BGE for 30 min. Before each injec-

tion, the capillary was rinsed for 5 min with 0.1 M NaOH, followed by water for 2 min and then equilibrated with running buffer for 8 min. The pH of solutions was measured using a Beckman Φ 350 pH meter (Fullerton, CA).

2.3. Sample preparation

A total of 10 wines (6 red and 4 white) were obtained from a local liquor store. The flavonoids were extracted from the wine samples before analysis. In the extraction, wine samples (15 mL) were mixed with 45 mL diethyl ether in a separatory funnel. The mixture was shaken for about 30 min and after extraction the ether layer was collected. The remaining aqueous layer was mixed with 45 mL ethyl acetate and was shaken again for several minutes to ensure complete extraction of flavonoids. After extraction, the ethyl acetate layer was collected and combined with the diethyl ether portion previously collected. The combined extract was dried with 5 g of anhydrous sodium sulphate for about 30 min. The dried solution was concentrated with rotary evaporation and taken to dryness under high vacuum. The dried extract was dissolved in 2 mL of 1-propanol/water (1:1) and then filtered with a 0.22 μm filter.

3. Results and discussion

3.1. Optimization of the MEKC separation conditions

The flavonoids were analyzed using a borate buffer containing SDS. Borate was employed because it can complex with the flavonoids to form more soluble complex anions (Volpi, 2004). The optimization and separation was achieved by optimizing the wavelength of UV detection, the pH of the buffer, borate concentration, organic solvent, and SDS concentration. Using a photodiode array detector, UV spectra of the flavonoids were obtained. Based on the spectra, the UV detection was investigated at 214 nm, 254 nm, and 280 nm for the flavonoids studied. The measurement at 214 nm yielded the best signal and a more stable background as well as the best separation for the flavonoids studied. Thus, 214 nm was chosen as the optimum UV detection wavelength throughout the experiment.

The acidity (pH) of the running buffer affects the electroosmotic flow (EOF) as well as the overall charge of the analytes, which determine the migration time and affect the separation of the analytes. The pH of the running buffer was varied from pH 8.0 to 10.0 in increments of 0.5. The optimum resolution was achieved when the running buffer pH is 9.0. Using 20% 1-propanol gave the best results for the analysis. The use of 1-propanol did not interfere with the analysis. The borate concentration was also studied and 40 mM was found to be the optimum concentration for the separation.

To improve the resolution, the sodium dodecyl sulfate (SDS) concentration present in the borate buffer was optimized. The SDS concentration was varied from 10 mM to 80 mM in a 40 mM borate running buffer.

A typical electropherogram for the six flavonoids separated using the selected optimum conditions is shown in Fig. 2. It can be seen that satisfactory separation is achieved within 16 min.

3.2. Regression equations, detection limits, recovery, and reproducibility for MEKC

Calibration curves for catechin (1), naringenin (2), kaempferol (3), apigenin (4), myricetin (5), and quercetin (6), ranging from 1.7 μg mL^{-1} to 200.0 μg mL^{-1} were established using the optimum separation conditions. In some cases, resorcinol was added in the separation as an internal standard to ensure the CE system is in proper conditions during these analyses. The detection limit is evaluated on the basis of S/N of 3. The results of the regression equations of calibration curves and detection limits for the six flavonoids are summarized in Table 1.

The flavonoids were identified by spiking the extracted wine sample with a known amount of each flavonoid standard. The recovery values obtained for a typical red wine are shown in Table 2.

The reproducibility of the MEKC analysis was established by injecting three injections of the same standard mixture. The coefficient of variation (CV) for the migration times and the peak areas were calculated and the results are listed in Table 3. The CV for the retention times of all the peaks of the six flavonoid standards was <2% and the CV for the peak area was <5%. Reproducibility obtained using repeated injections of the wine samples also gave comparable results. This indicates that this MEKC method is highly reproducible.

3.3. Wine sample analysis

Under the optimum conditions, flavonoids were determined in several red and white wine samples. Typical

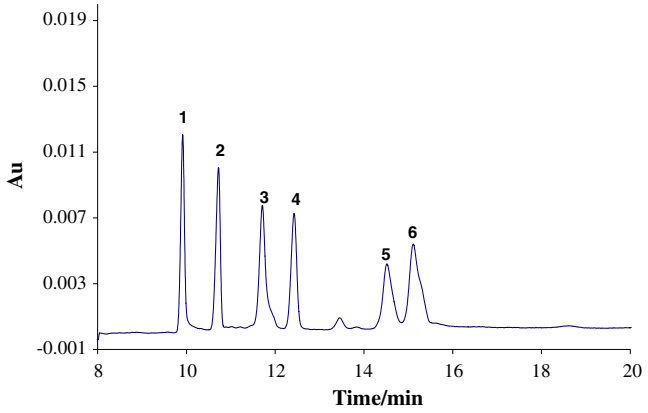

Fig. 2. Electropherogram obtained from a standard mixture of six flavonoid compounds of 3.3×10^{-5} g mL^{-1} of catechin (1), naringenin (2), kaempferol (3), apigenin (4), myricetin (5), and quercetin (6). Optimized separation condition was 40 mM borate solution containing 40 mM SDS at pH 9.0.

Table 1
Regression analysis on calibration curves, recovery, and detection limits

Compound	Regression equation	Correlation coefficient (%)	Linear range (μg mL^{-1})	Detection limit (μg mL^{-1})
Catechin	$y = 724.6x + 4687.9$	99.4	1.7–200.0	0.0226
Naringenin	$y = 708.4x - 198.4$	99.6	1.7–200.0	0.0231
Kaempferol	$y = 825.7x - 2246.1$	98.2	3.3–200.0	0.0198
Apigenin	$y = 1104.2x - 4746.0$	99.3	3.3–200.0	0.0148
Myricetin	$y = 814.4x - 13268.0$	99.0	10–200.0	0.0201
Quercetin	$y = 734.5x - 2729.3$	99.6	6.7–200.0	0.0223

Table 2
Results of the recovery for this MEKC method ($n = 3$)

Compound	Original amount (μg mL^{-1})	Added amount (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)	RSD (%)
Catechin	15.3	60.0	69.7	93.0	0.9
Naringenin	8.0	40.0	41.0	85.0	1.3
Kaempferol	13.0	10.0	15.6	68.0	2.7
Apigenin	7.8	20.0	20.3	73.0	1.3
Myricetin	20.8	20.0	37.0	91.0	6.6
Quercetin	10.3	20.0	27.9	92.0	5.6

Table 3
Reproducibility of the studied flavonoids using MEKC ($n = 3$)

Flavonoid	Migration time (min)	Migration time, CV (%)	Peak area, CV (%)
Catechin	10.1	1.3	2.8
Naringenin	10.9	1.5	2.2
Kaempferol	11.9	1.6	0.9
Apigenin	12.6	1.4	0.4
Myricetin	14.8	1.6	6.1
Quercetin	15.4	1.4	4.8

electropherograms for a red wine and a white wine are shown in Fig. 3. By comparing the migration time of analytes with the electropherogram of a standard mixture, and by spiking of the wine samples, the six flavonoids can be determined. Analyzing the original wine directly did not yield accurate results as the electropherogram had a large background. It was therefore difficult to quantify accurately the peak areas of the analytes. The background improved dramatically following the extraction procedure used in this study and made quantification possible. The assay results for extracted samples of 6 red wines (labeled R1–R6) and 4 white wines (labeled W1–W4) are listed in Table 4. Three injections were made for each wine sample. The actual concentrations of the six flavonoids in the original wines would be 2/15 of the concentration values of the wine extracts listed in Table 4.

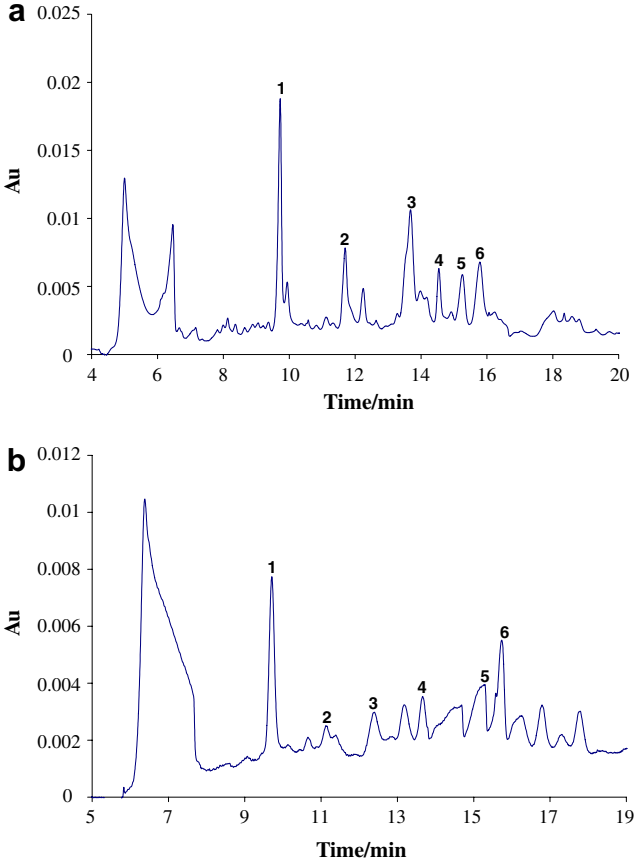

Fig. 3. (a) and (b) are electropherograms obtained from diluted extracts of a red wine and a white wine respectively under optimum conditions.

4. Conclusions

An MEKC method was developed for the analysis of flavonoids with possible anti-carcinogenic effects. The compounds were separated in 16 min in the BGE consisting of 40 mM borate containing 40 mM SDS and 20% v/v 1-propanol. This paper describes a practical method that can be used to compare the amount of potentially anti-carcinogenic flavonoids, and can be used for fingerprinting of wines from different regions.

Table 4
Assay results for the flavonoids in 10 wine samples in μg mL^{-1} ($n = 3$)

	Catechin	Naringenin	Kaempferol	Apigenin	Myricetin	Quercetin
R1	56.7	78.6	32.0	22.4	30.3	26.0
R2	68.9	162.4	42.4	46.4	190.7	35.0
R3	121.7	70.6	13.5	ND	33.7	27.6
R4	24.4	88.8	111.1	33.3	63.3	33.1
R5	63.3	30.7	94.0	32.7	27.3	ND
R6	22.2	83.5	61.3	ND	85.4	50.0
W1	85.4	17.0	ND	30.0	30.9	14.6
W2	77.2	7.9	24.6	17.5	58.8	46.2
W3	78.3	52.6	28.7	ND	60.9	80.0
W4	39.9	7.7	33.9	24.5	61.2	46.3

Data in the table are the means of three replicates; ND: not detected.

Acknowledgements

Financial support by the Thompson Rivers University Scholarly Activity Program and the Department of Chemistry, University of British Columbia are gratefully acknowledged.

References

Bayard, V., Chamorro, F., Motta, J., & Hollenberg, N. K. (2007). Does flavanol intake influence mortality from nitric oxide-dependent processes? Ischemic heart disease, stroke, diabetes mellitus, and cancer in Panama. *International Journal of Medical Sciences, 4*(1), 53–58.

Bilbao, M. de L. M., Andres-Lacueva, C., Jauregui, O., & Lamuela-Raventos, R. M. (2007). Determination of flavonoids in a citrus fruit extract by LC-DAD and LC–MS. *Food Chemistry, 101*, 1742–1747.

Brown, D. E., Rashotte, A. M., Murphy, A. S., Normanly, J., Tague, B. W., Peer, W. A., et al. (2001). Flavonoids act as negative regulators of auxin transport in vivo in *Arabidopsis*. *Plant Physiology, 126*, 524–535.

Dadakova, E., Prochazkova, E., & Krizek, M. (2001). Application of micellar electrokinetic capillary chromatography for quantitative analysis of quercetin in plant materials. *Electrophoresis, 22*, 1573–1578.

Da Queija, C., Queiros, M. A., & Rodrigues, L. M. (2001). Determination of flavonoids in wine by high performance liquid chromatography. *Journal of Chemical Education, 78*, 236–237.

De Rijke, E., Out, P., Niessen, W. M. A., Ariese, F., Gooijer, C., & Brinkman, U. A. T. (2006). Analytical separation and detection methods for flavonoids. *Journal of Chromatography A, 1112*, 31–63.

Fang, F., Li, J-M., Pan, Q-H., & Huang, W-D. (2007). Determination of red wine flavonoids by HPLC and effect of aging. *Food Chemistry, 101*, 428–433.

Fiamegos, Y. C., Nanos, C. G., Vervoort, J., & Stalikas, C. D. (2004). Analytical procedure for the in-vial derivatization–extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by gas chromatography with mass-selective detection. *Journal of Chromatography A, 1041*, 11–18.

Furusawa, M., Tanaka, T., Ito, T., Nishikawa, A., Yamazaki, N., Nakaya, K., et al. (2005). Antioxidant activity of hydroxyflavonoids. *Journal of Health Sciences, 51*(3), 376–378.

Georgetti, S. R., Casagrande, R., Di Mambro, V. M., Azzolini, A. E., & Maria, J. (2003). Evaluation of the antioxidant activity of different flavonoids by the chemiluminescence method. *American Association of Pharmaceutical Scientists Journal, 5*(2), 1–4.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology and Therapeutics, 96*, 67–202.

Ielpo, M. T. L., Basile, A., Miranda, R., Moscatiello, V., Nappo, C., Sorbo, S., et al. (2000). Immunopharmacological properties of flavonoids. *Fitoterapia, 71*, S101–S109.

Mak, P., Leung, Y.-K., Tang, W.-Y., Harwood, C., & Ho, S.-M. (2006). Apigenin suppresses cancer cell growth through ERβ. *Neoplasia, 8*(11), 896–904.

Michael, G. L., Hertog, P. H., Hollman, P., & Dini, P. (1992). Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *Journal of Agricultural and Food Chemistry, 40*, 1591–1598.

Middleton, E., Jr., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*, 673–751.

Miean, K. H., & Mohamed, S. (2001). Flavonoid (Myricetin, Quercetin, Kaempferol, Luteolin, and Apigenin) Content of Edible Tropical Plants. *Journal of Agricultural and Food Chemistry, 49*, 3106–3112.

Molnar-Perl, I., & Fuzfai, Zs. (2005). Chromatographic, capillary electrophoretic and capillary electrochromatographic techniques in the analysis of flavonoids. *Journal of Chromatography A, 1073*, 201–227.

Powles, T. (2004). Isoflavones and women's health. *Breast Cancer Research, 6*(3), 140–142.

Proestos, C., Boziaris, I. S., Nychas, G.-J. E., & Komaitis, M. (2006). Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chemistry, 95*, 664–671.

Rehova, L., Skerikova, V., & Jandera, P. (2004). Optimisation of gradient HPLC analysis of phenolic compounds and flavonoids in beer using a CoulArray detector. *Journal of Separation Science, 27*, 1345–1359.

Rodriguez-Delgado, M. A., Perez, M. L., Corbella, R., Gonzalez, G., & Montelongo, F. J. (2000). Optimization of the separation of phenolic compounds by micellar electrokinetic capillary chromatography. *Journal of Chromatography A, 871*, 427–438.

Soczewinski, E., Hawryl, M. A., & Hawryl, A. (2001). Retention behaviour of some flavonoids in 2D-TLC systems on cyano bonded polar phases. *Chromatographia, 54*, 789–794.

Suntornsuk, L. (2002). Capillary electrophoresis of phytochemical substances. *Journal of Pharmaceutical and Biomedical Analysis, 27*, 679–698.

Tonin, F. G., Jager, A. V., Micke, G. A., Farah, J. P. S., & Tavares, M. F. M. (2005). Optimization of the separation of flavonoids using solvent-modified micellar electrokinetic chromatography. *Electrophoresis, 26*, 3387–3396.

Volpi, N. (2004). Separation of flavonoids and phenolic acids from propolis by capillary zone electrophoresis. *Electrophoresis, 25*, 1872–1878.

Wang, S-P., & Huang, K-J. (2004). Determination of flavonoids by high-performance liquid chromatography and capillary electrophoresis. *Journal of Chromatography A, 1032*, 273–279.

Weinberger, R. (2000). *Practical Capillary Electrophoresis* (2nd ed.). New York: Academic Press.

Wu, T., Guan, Y., & Ye, J. (2007). Determination of flavonoids and ascorbic acid in grapefruit peel and juice by capillary electrophoresis with electrochemical detection. *Food Chemistry, 100*, 1573–1579.

Yang, B., Kotani, A., Arai, K., & Kusu, F. (2001). Estimation of the antioxidant activities of flavonoids from their oxidation potentials. *Analytical Sciences, 17*, 599–604.