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Journal of Chromatography A, 923 (2001) 255–262

JOURNAL OF CHROMATOGRAPHY A

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Determination of puerarin, daidzein and rutin in *Pueraria lobata* (Wild.) Ohwi by capillary electrophoresis with electrochemical detection

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Received 27 February 2001; received in revised form 16 May 2001; accepted 21 May 2001

Abstract

A method based on capillary electrophoresis with electrochemical detection was developed for the determination of puerarin, daidzein and rutin. Effects of several important factors such as the acidity and concentration of running buffer, separation voltage, injection time, and detection potential were investigated to acquire the optimum conditions. The working electrode was a 300-μm diameter carbon disc electrode positioned opposite the outlet of capillary. The three analytes could be well separated within 12 min in a 40 cm length capillary at a separation voltage of 9 kV in a 50 mmol/l borate buffer (pH 9.0). The relationship between peak currents and analyte concentrations was linear over about three orders of magnitude with detection limits (S/N=3) ranging from $0.241\cdot10^{-6}$ to $0.511\cdot10^{-6}$ mol/l for all compounds. This proposed method demonstrated long-term stability and reproducibility with relative standard deviations of less than 5% for both migration time and peak current (n=7). It has been successfully applied for the determination of puerarin, daidzein and rutin in Chinese traditional drugs, the vines of *Pueraria lobata* (Wild.) Ohwi and Puerariae Radix. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Pharmaceutical analysis; *Pueraria lobata*; Puerarin; Daidzein; Rutin; Isoflavones; Aglycones

1. Introduction

Chinese traditional medicine, Pueraria Radix, is the root of *Pueraria lobata* (Wild.) Ohwi. It is an important Chinese crude herb used to therapy shoulder or wrist stiffen, common cold, influenza, vascular hypertension, etc. [1]. Puerarin and daidzein are two active isoflavones in it that is the main material for extracting puerarin for medicinal industry in China [2]. Daidzein is the aglycone of puerarin and has been synthesized artificially. Both of them have been used clinically as therapeutical medicines [2]. Isoflavones (including puerarin and daidzein) from Pueraria Radix have many important physiological activities such as antiproliferative effects on human cancer cell lines [3], inhibiting alcohol dehydrogenase and xanthine oxidase [4,5]. Recent investigations demonstrate that puerarin is an effective antioxidant and shows effects against glutamate excitotoxicity on cultured mouse cerebral cortical neurons [6,7]. The latest pharmacological studies indicate that daidzein has antigiardial activity [8],

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PII: S0021-9673(01)00996-7

antioxidant action and potential antidiabetic properties [9,10].

Our preliminary experiment shows that rutin also presents in Puerariae Radix, so it is determined in this paper simultaneously. Rutin is an important flavonoid with diverse physiological activities [11,12]. In China, the Public Health Department requires that the content of puerarin in Puerariae Radix should not be less than 2.4% [1]. Moreover, some concentrated composite herbal preparations that contain Puerariae Radix in their prescriptions are widely used in oriental countries for their conveniences of use. Hence, it is interesting to establish some simple, economical and accurate methods for the determination of puerarin, daidzein and rutin in Chinese traditional medicines.

Liquid chromatography (LC) has been widely used for the individual and simultaneous determination of puerarin, daidzein and other flavonoids in Puerariae Radix [13–15], composite herbal preparations with Puerariae Radix as their ingredients [14,16,17] and human urine [18,19]. Pulse porlarography [20] has also been applied for the determination of flavonoids in Puerariae Radix.

As naturally occurring polyphenols, most flavonoids are electroactive at modest oxidation potential, electrochemical methods are useful for their analysis [21–23]. Yoshimi et al. have determined isoflavones (including puerarin and daidzein) in Puerariae Radix by LC with amperometric detection [15]. In a previous report [24], we have differentiated Scutellariae Radix from Astragali Radix by determining baicalein and baicalin (two bioactive flavonoids) contents in them by capillary electrophoresis with electrochemical detection (CE–ED). Moreover, CE–ED has also been employed for the determination of several flavonoids in Scutellariae Radix and its pharmaceutical preparation with satisfactory assay results [25].

As puerarin, daidzein and rutin are electroactive at the carbon electrode [11,12,15], CE–ED should be an assistant, alternative and complement technique for the constituent investigation of crude drugs. Moreover, ED can also provide higher selectivity as only electroactive substances can be detected so that the electropherograms are simplified, which is important for the analysis of medicinal plants as the constituents in them are usually complex. CE has already been employed for the determination of puerarin and some other flavonoids in Puerariae Radix and its pharmaceutical preparation coupled with a UV detector [26].

In this study, puerarin, daidzein and rutin (their molecular structures are shown in Fig. 1) in Chinese traditional herbal drugs, the vines of *Pueraria lobata* (Wild.) Ohwi and Puerariae Radix, were determined by CE–ED. This method is simple, sensitive, reliable and efficient providing not only a way for evaluating the quality of Puerariae Radix in the marketplace, but also an excellent alternative method in quality

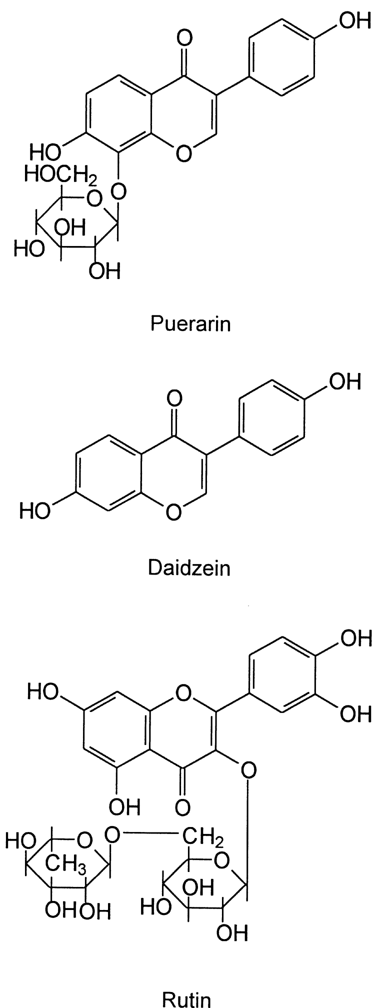


Fig. 1. The molecular structures of puerarin, daidzein and rutin.

control for medicinal manufacturers and the constituent analysis of plants.

2. Experimental

2.1. Reagent and solutions

Both puerarin and rutin were purchased from Fluka (Buchs, Switzerland). Daidzein was obtained from Sigma (St. Louis, MO, USA). All aqueous solutions were made up in doubly distilled water. Other chemicals were of analytical grade.

Stock solutions of puerarin, daidzein and rutin ($5.0\cdot10^{-3}$ mol/l) were prepared in 50% (v/v) ethanol in 50 mmol/l borate buffer (pH 8.0) mixtures and diluted to the desired concentration with the running buffer just prior to use. The solutions were kept in a 4°C refrigerator and were stable for at least 1 month. Borate in the stock solution was used to enhance the solubility of the investigated analytes.

2.2. Apparatus

The CE–ED system has been described previously [27]. A ±30 kV high-voltage d.c. power supply (Shanghai Institute of Nuclear Research, China) provided a separation voltage between the ends of the capillary. The inlet end of the capillary was held at a positive potential and the outlet end of capillary was maintained at ground. The separations were proceeded in a 40 cm×25 μm I.D.×360 μm O.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA).

A three-electrode electrochemical cell consisting of a 300 μm diameter carbon disc working electrode, a platinum auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode, was used in combination with a BAS LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA). The filter of the detector was set at 0.1 Hz. The working electrode was positioned carefully opposite the outlet of the capillary with the aid of a micromanipulator (Correct, Tokyo, Japan) and arranged in a wall-jet configuration [28]. The distance between the tip of the working electrode and the capillary outlet was as close as possible so that the CE effluent directly impinged upon the electrode surface. The electropherograms were recorded using a LKB·REC 1 chart record (Pharmacia, Sweden). An YS 38-1000 220 V alternate constant-voltage power supply (Shanghai Instrumental Transformer Factory, Shanghai, China) was employed to suppress the voltage fluctuation of the power line. The whole system was assembled in a 10 m^2 Faraday room that was air-conditioned at 20°C in order to minimize the effects of external noise sources.

2.3. Sample preparation

Samples 1 and 2 of Puerariae Radix were obtained from Huangshan Crude Drug Co. (Huangshan, China) and Sun-Tian-Tang Chinese Traditional Drug Store (Shanghai, China), respectively. The vines of *Pueraria lobata* (Wild.) Ohwi were collected from the east campus of Medical Center of Fudan University (Shanghai, China) and dried in the air. All the plant samples were kindly identified by Professor D. Chen (Department of Pharmacognosy, Medical Center of Fudan University, Shanghai, China). They were all dried at 60°C for 4 h in an oven and then were gently pulverized. An accurate weighed amount of the powder (about 2 g) was refluxed with 50 ml of 95% ethanol for 3 h in an 80°C water bath according to the pharmacopeial method [1]. After cooling, the mixture was filtered through a paper filter and the residues were washed twice with 10 ml of 95% ethanol. The extract and washings were combined and concentrated to about 40 ml under vacuum and then diluted to 50 ml in a volumetric flask with 95% ethanol. The extracts were further diluted with the running buffer at ratio of 10 just prior to their analysis. Peak identification was performed by standard addition method.

2.4. Procedures

Before use, the carbon disc electrode was successively polished with emery paper and alumina powder, sonicated in doubly distilled water. CE was performed at the separation voltage of 9 kV with a 50 mmol/l borate buffer (BB, pH 9.0) used as running buffer. The potential applied to the working electrode was 0.90 V (versus SCE). Samples were injected electrokinetically at 9 kV for 6 s. Moreover, sample solutions, standard solutions and the running

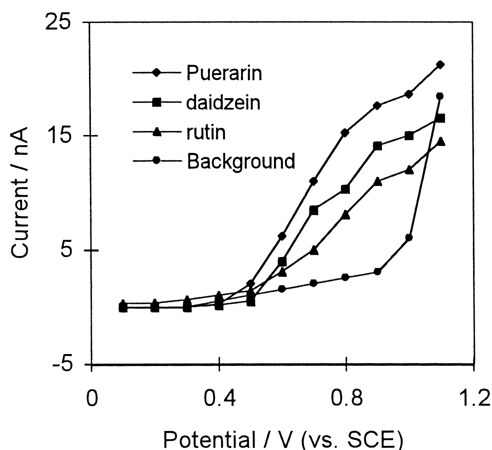


Fig. 2. Hydrodynamic voltammograms (HDVs) of $2.0 \cdot 10^{-4}$ mol/l of puerarin, $1.0 \cdot 10^{-4}$ mol/l of daidzein and $2.0 \cdot 10^{-4}$ mol/l of rutin in CE. Fused-silica capillary: 40 cm×25 μm I.D.; working electrode: 300 μm diameter carbon disk electrode; running buffer: 50 mmol/l BB (pH 9.0); separation voltage: 9 kV; electrokinetic injection: 6 s (at 9 kV).

buffer were all filtered through a syringe cellulose acetate filter (0.22 μm) prior to their use.

3. Results and discussion

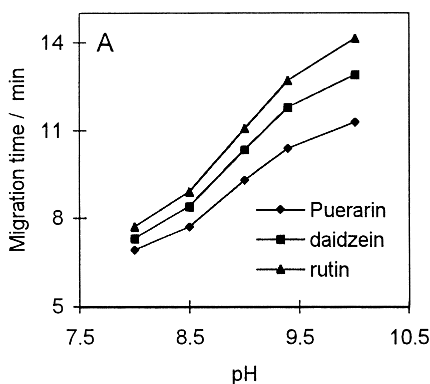
3.1. Effect of the potentials applied to the working electrode

Since puerarin, daidzein and rutin can be oxidized electrochemically at a relatively moderate potential [11,12,15], electrochemical detection was used in this work. Hydrodynamic voltammograms (HDVs) of puerarin, daidzein and rutin are illustrated in Fig. 2. When the applied potential exceeds +0.60 V (versus SCE), the peak current of all analytes increases rapidly. However, the current response increases slower when the potential passes above +0.90 V (versus SCE). Although an applied potential greater than +0.90 V (versus SCE) results in higher peak currents, both the baseline noise and the background current increase strongly resulting in an unstable baseline. Thus, the applied potential of the working electrode was maintained at +0.90 V (versus SCE), where the background current is not too high and the *S/N* ratio is the highest. The working electrodes showed good stability and high reproducibility at the optimum potential for more than 3 weeks.

3.2. Effects of the pH and concentration of the running buffer

In order to improve the resolution and solubility of analytes, alkaline borate buffer was employed in this study as puerarin and rutin can form negative-charged complexes with boric acid in alkaline solution [29].

The acidity of the running buffer affect the ζ-potential, the electroosmotic flow (EOF) as well as the overall charge of the analytes, which determine the migration time and the separation of the analytes [30]. The effect of the running buffer pH on the migration time of the analytes is shown in Fig. 3A.



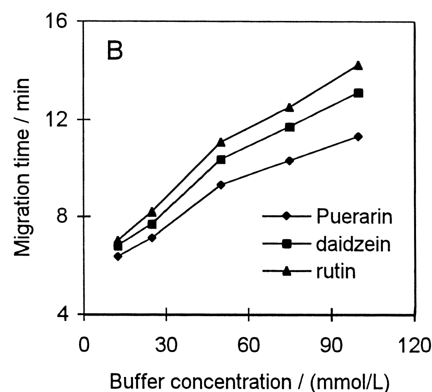


Fig. 3. Effect of (A) acidity and (B) concentration of the running buffer on the migration time of the analytes. Working potential: +0.90 V (versus SCE); other conditions as in Fig. 2.

The running buffers were 50 mmol/l BBs at five different pH values (8.0, 8.5, 9.0, 9.4 and 10.0). As shown in Fig. 3A, the resolution of the three analytes is poor at pH 8.0. When the running buffer pH increases, the migration time increases with the resolution improved due to the dissociation of the hydroxyl groups for all analytes. Meanwhile, the peak current is low and the peak shape becomes poor at pH value above 9.5. At pH 9.0, the three analytes can be well separated within a relatively short time. In this experiment, 50 mmol/l BB with pH 9.0 is chosen as the running buffer in considering the peak current, resolution and the analytical time.

As the buffer concentration influences the viscosity coefficient of the solution, the diffusion coefficient of analytes and the ζ-potential of the inner surface of capillary tube as well, it affects not only the resolution and migration time of the analytes, but also the peak current. Fig. 3B indicates that the migration time and the resolution increases with increasing buffer concentration. However, higher buffer concentrations (>50 mmol/l) also have a negative effect on the detection limits because the peak currents of all analytes decrease and the effect of Joule heat becomes more pronounced. So 50 mmol/l BB (pH 9.0) is chosen as the running buffer in this work in considering the peak current, resolution, analytical time and the buffer capacity.

3.3. Effect of separation voltage and injection time

Increasing the voltage gives shorter migration time for all compounds, but also increases the baseline noise, resulting in poorer detection limits. It is found that higher separation voltages are not beneficial to the resolution of all three compounds. Moreover, higher separation voltages may result in higher Joule heat that directly affects the separation efficiency of this method. However, too low separation voltages will increase the analysis time considerably, which in turn cause peak broadening. Based on experiments, 9 kV was chosen as the optimum voltage to accomplish a good compromise.

The effect of injection time on CE separation was investigated by changing the sampling time (2, 4, 6, 8 and 10 s at a voltage of 9 kV). It was found that both the peak current and the peak width increase with increasing sampling time. When injection time exceeds 6 s, the peak current increases slower and peak broadening becomes more severe. In this experiment, 6 s (at 9 kV) is selected as the optimum injection time in considering the resolution and sensitivity.

Through the experiments above, the optimum conditions for determining puerarin, daidzein and rutin are acquired. A typical electropherogram for a standard mixture solution is shown in Fig. 4A. Baseline separation for all three analytes can be achieved within 12 min.

3.4. Reproducibility, linearity and detection limits of puerarin, daidzein and rutin

A standard mixture solution of $2.0 \cdot 10^{-4}$ mol/l of puerarin, $1.0 \cdot 10^{-4}$ mol/l of daidzein and $2.0 \cdot 10^{-4}$ mol/l of rutin was analyzed seven times every 30 min to determine the reproducibility of the peak current and migration time for all analytes under the optimum conditions. The relative standard deviations (RSDs) of peak current and migration time are 2.71% and 0.867% for puerarin, 2.47% and 1.54% for daidzein, 4.21% and 0.936% for rutin, respectively.

A series of the standard mixture solutions of puerarin, daidzein and rutin with concentration ranging from $1.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-3}$ mol/l were tested to determine the linearity for all analytes at the carbon disc electrode in this method. The results of regression analysis on calibration curves and detection limits are presented in Table 1. The determination limits are evaluated on the basis of a signal-to-noise ratio of 3. The calibration curves exhibit satisfactory linear behavior over the concentration range of about three orders of magnitude with the detection limits ranging from $0.241 \cdot 10^{-6}$ to $0.511 \cdot 10^{-6}$ mol/l for all analytes.

3.5. Application and recovery

Under the optimum conditions, CE–ED was applied for the determination of puerarin, daidzein and rutin in Chinese traditional drugs, the vines of *Pueraria lobata* (Wild.) Ohwi and Puerariae Radix according to the procedures described above. Typical electropherograms of the diluted extracts are shown in Fig. 4B–D. Assay results are summarized in Table

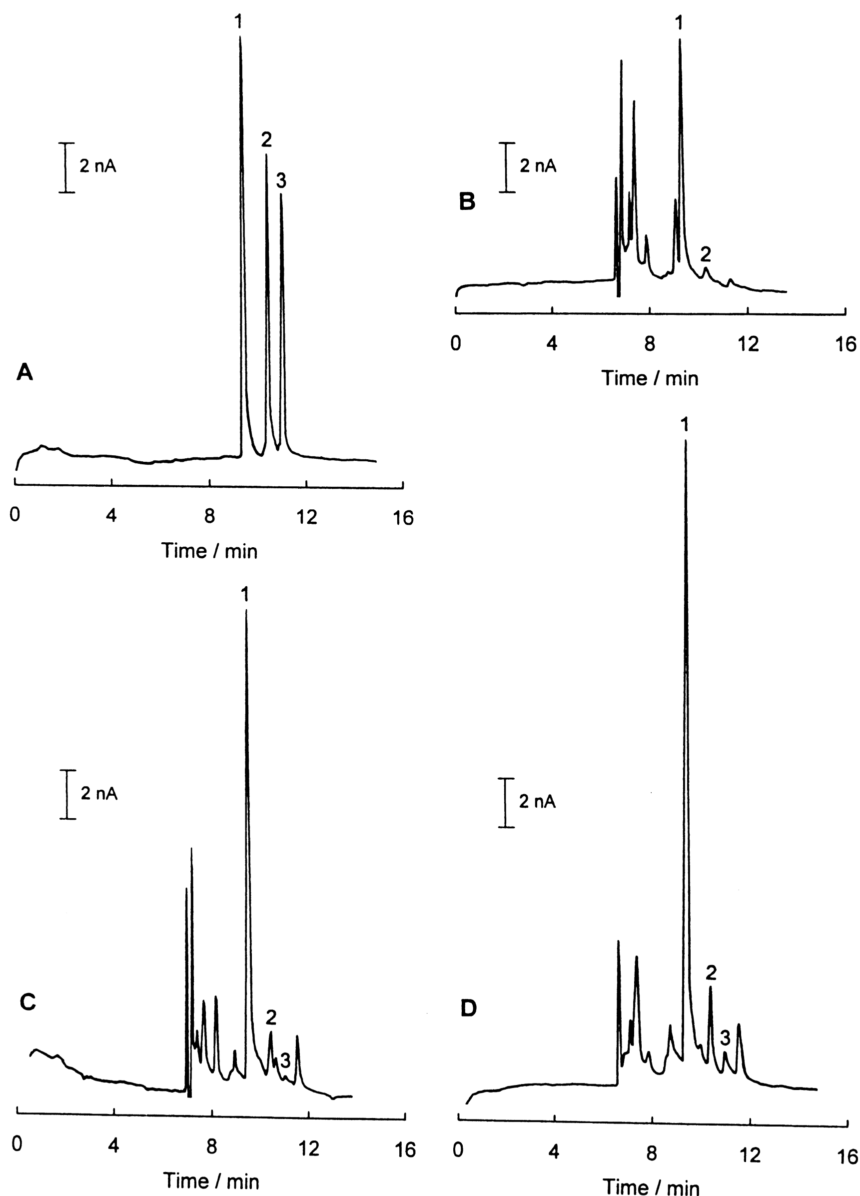


Fig. 4. Electropherogram of (A) the standard mixture solution of $2.0 \cdot 10^{-4}$ mol/l of puerarin, $1.0 \cdot 10^{-4}$ mol/l of daidzein and $2.0 \cdot 10^{-4}$ mol/l of rutin and the typical electropherograms of the diluted extracts from (B) the vines of *Pueraria lobata* (Wild.) Ohwi, (C) Puerariae Radix 1 and (D) Puerariae Radix 2 under the optimum conditions. Working potential: +0.90 V (versus SCE); other conditions as in Fig. 2. Peaks: 1=puerarin, 2=daidzein, 3=rutin.

2. The puerarin and daidzein contents in Puerariae Radix are similar to the previous reports [13,15,29]. As the vine of *Pueraria lobata* (Wild.) Ohwi reproduces faster than the root and is easy to be collected without digging earth, it can be recommended as the material for extracting puerarin although puerarin content in it is relatively low. More importantly, it is beneficial to the conservation of forests.

By comparing Fig. 4C–D with the electrophero-

Table 1
The results of regression analysis on calibration curves and the detection limits[a]

Compound	Regression equation $y=a+bx$[b]	Correlation coefficient	Linear range ($\cdot 10^{-3}$ mol/l)	Detection limit[c] ($\cdot 10^{-6}$ mol/l)
Puerarin	$y=-0.1057+87.221x$	0.9996	0.0025–1.0	0.344
Daidzein	$y=0.0859+124.728x$	0.9995	0.0010–1.0	0.241
Rutin	$y=-0.1736+58.654x$	0.9993	0.0050–1.0	0.511

[a] Working potential is 0.90 V (versus SCE). Other conditions as in Fig. 2.
[b] y and x are the peak current (nA) and concentration of the analytes ($\cdot 10^{-3}$ mol/l), respectively.
[c] The detection limits corresponding to concentrations giving signal-to-noise ratio of 3.

Table 2
Assay results of the analytes in plant samples ($n=3$, mg/g)[a]

Sample	Puerarin	Daidzein	Rutin
The vines of *Pueraria lobata* (Wild.) Ohwi	11.378 (3.81)[b]	0.5911 (4.29)	N.F.[c]
Puerariae Radix 1	23.361 (2.45)	0.9784 (3.38)	0.5665 (5.17)
Puerariae Radix 2	30.745 (2.21)	1.6816 (3.10)	2.6059 (4.15)

[a] Working potential is 0.90 V (versus SCE). Other conditions as in Fig. 2.
[b] The data in parentheses are RSDs (%).
[c] N.F.=Not found.

grams of CE–UV for Puerariae Radix [29], it can be found that the electropherograms based on CE–ED have been simplified as only electroactive species can be detected on the electrode. Moreover, this method is an ideal technique for determining puerarin in Puerariae Radix as the peak of puerarin is the highest in the electropherograms.

Accurate amounts of puerarin, daidzein and rutin were added to the diluted extract of Puerariae Radix in the running buffer, and the recovery values were obtained using their peak currents from the calibration curve under the same conditions. The average recoveries and RSDs for puerarin, daidzein and rutin are listed in Table 3. The results indicate that this method is accurate and rugged for all analytes.

In order to improve the migration time and peak current reproducibility between consecutive analysis of practical samples, the capillary was washed with 0.1 mol/l NaOH for 5 min, and then was conditioned with the running buffer for at least 5 min electrokinetically at 9 kV. The electrochemical detection used in this work can provide a high selectivity as only electroactive substances can be detected. The interferences of glucose and sucrose were not found, as it could not be oxidized on the carbon electrode under the optimum conditions.

Acknowledgements

The authors are grateful for the financial support provided by the Natural Science Foundation of China (grant No. 20075008) and the Med-X foundation of Fudan University.

Table 3
Determination results of the recovery for this method ($n=3$)[a]

Compound	Original amount ($\cdot 10^{-6}$ mol/l)	Added amount ($\cdot 10^{-6}$ mol/l)	Found amount ($\cdot 10^{-6}$ mol/l)	Recovery (%)	RSD (%)
Puerarin	291.21	100.00	389.84	98.63	2.67
Daidzein	26.48	25.00	50.70	96.88	3.75
Rutin	16.36	25.00	40.69	97.32	4.12

[a] Working potential is 0.90 V (versus SCE). Other conditions as in Fig. 2.

References

[1] The Public Health Department of People's Republic of China, in: Pharmacopoeia of the People's Republic of China, Vol. 1, Chemical Industry Press, Beijing, 2000, p. 273.

[2] W.J. Sun, J.F. Sheng, in: Handbook of Natural Active Constituents, Chinese Medicinal Science and Technology Press, Beijing, 1998, p. 165;
W.J. Sun, J.F. Sheng, in: Handbook of Natural Active Constituents, Chinese Medicinal Science and Technology Press, Beijing, 1998, p. 472.

[3] K. Yanagihara, A. Ito, T. Toge, Numoto, Cancer Res. 53 (1993) 5815.

[4] W.M. Keung, Alcohol Clin. Exp. Res. 17 (1993) 1254.

[5] W.S. Chang, Y.J. Lee, F.J. Lu, H.C. Chiang, Anticancer Res. 13 (1993) 2165.

[6] M.C. Guerra, E. Speroni, M. Broccoli, M. Cangini, P. Pasini, A. Minghett, N. Crespi-Perellino, M. Mirasoli, G. Cantelli-Forti, M. Paolini, Life Sci. 67 (2000) 2997.

[7] L.P. Dong, T.Y. Wang, Acta Pharm. Sinica 19 (1998) 339.

[8] I.A. Khan, M.A. Avery, C.L. Burandt, D.K. Goins, J.R. Mikell, T.E. Nash, A. Azadegan, L.A. Walker, J. Nat. Prod. 63 (2000) 1414.

[9] A. Arora, M.G. Nair, G.M. Strasburg, Arch. Biochem. Biophys. 356 (1998) 133.

[10] K. Vedavanam, S. Srijayanta, J. O'Reilly, A. Raman, H. Wiseman, Phytother. Res. 13 (1999) 601.

[11] G. Chen, X.H. Ding, J.N. Ye, Chem. J. Chin. Univ. 21 (2000) 1364.

[12] G. Chen, H.W. Zhang, J.N. Ye, Anal. Chim. Acta 423 (2000) 69.

[13] H.J. Song, M. Zeng, J.H. Hu, D.Y. Wang, H.M. Zhang, Chin. J. Pharm. Anal. 20 (2000) 223.

[14] X. Zhang, F. Zhou, Y. Yan, L. Chen, Chin. J. Chin. Mater. Med. 20 (1995) 477;
X. Zhang, F. Zhou, Y. Yan, L. Chen, Chin. J. Chin. Mater. Med. 20 (1995) 512.

[15] K. Yoshimi, M. Munehiko, U. Yasuyuki, J. Chromatogr. 347 (1985) 438.

[16] N. Okamura, H. Miki, H. Orii, Y. Masaoka, S. Yamashita, H. Kobayashi, A. Yagi, J. Pharm. Biomed. Anal. 19 (1999) 603.

[17] S. Tan, X. Qiu, G. Li, B. Zhao, Y.R. Liang, Chin. J. Chin. Mater. Med. 21 (1996) 732.

[18] C.O. Cimino, S.R. Shelnutt, M.J. Ronis, T.M. Badger, Clin. Chim. Acta 287 (1999) 69.

[19] A. A Franke, L.J. Custer, J. Chromatogr. B 662 (1994) 47.

[20] L.X. Xu, A.R. Liu, X.Q. Zhang, Acta Pharm. Sinica 22 (1987) 208.

[21] S.M. Lunte, J. Chromatogr. 384 (1987) 371.

[22] H.P. Hendrickson, A.D. Kaufman, C.E. Lunte, J. Pharm. Biomed. Anal. 12 (1994) 325.

[23] H.P. Hendrickson, M. Sahafayen, M.A. Bell, A.D. Kaufman, M.E. Hadwiger, C.E. Lunte, J. Pharm. Biomed. Anal. 12 (1994) 335.

[24] G. Chen, X.Y. Ying, J.N. Ye, Analyst 125 (2000) 815.

[25] G. Chen, H.W. Zhang, J.N. Ye, Talanta 53 (2000) 471.

[26] C.Y. Wang, H.Y. Huang, K.L. Kuo, Y.Z. Hsieh, J. Chromatogr. A 802 (1998) 225.

[27] G. Chen, J.N. Ye, J.S. Cheng, Chromatographia 52 (2000) 137.

[28] J.N. Ye, R.P. Baidwin, Anal. Chem. 65 (1993) 3525.

[29] Ph. Morin, F. Villard, M. Dreux, J. Chromatogr. 628 (1993) 161.

[30] Y.Z. Deng, J.L. He, in: High Performance Capillary Electrophoresis, Academic Press, Beijing, 1995, p. 26.