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Separation of coumarins by micellar electrokinetic capillary chromatography

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Abstract

Nine coumarins were successfully separated simultaneously using micellar electrokinetic capillary chromatography with 4-hydroxybenzoic acid as an internal standard. A carrier composed of buffer solution (20 m*M* sodium dodecyl sulfate–25 m*M* sodium borate–25 m*M* sodium dihydrogenphosphate)–acetonitrile (24:1) was found to be the most suitable electrolyte for this separation. The analysis time (22 min) was shorter than that using high-performance liquid chromatography (47 min). Contents of coumarins in the crude drug of Angelicae Tuhou Radix could be easily determined by the proposed method.

1. Introduction

Coumarins commonly exist in over 100 plant families, predominantly among the higher plants [1]. It is common to encounter species, especially the Guttiferae, Rutaceae and Umbelliferae, that contain 10, 20, or even more coumarins and of which four or five from the same species have often been reported. Angelicae Tuhou Radix is derived from the root of Umbelliferae plants, including *Angelica laxifora* Diels, *A. megaphylla* Diels and *A. pubescens* Maxim. [2]. Coumarins are their major components and about 20 kinds have been identified [3–15]. Several methods have been established to determine one or two coumarins contained in this crude drug, such as TLC–densitometry [16], high-performance liquid chromatography (HPLC) [17] and gas chromatography [18].

Micellar electrokinetic capillary chromatography (MEKC) was first reported by Terabe et al. [19] in 1984, and has been applied successfully to both charged and neutral compounds of some Chinese herbs [20,21]. Here we describe an application of MEKC to the separation of the coumarins most commonly contained in Angelicae Tuhou Radix, such as coumarin (**1**), umbelliferone (**2**), columbianetin (**3**), psoralen (**4**), xanthotoxin (**5**), bergapten (**6**), columbianetin acetate (**7**), osthol (**8**) and columbianadin (**9**) (Fig. 1). An HPLC method for simultaneous determination of these nine coumarins was also developed and compared.

2. Experimental

2.1. Reagents and materials

Columbianetin, columbianetin acetate, osthol and columbianadin were isolated from Angelicae Tuhou Radix [6,10]. Umbelliferone, coumarin,

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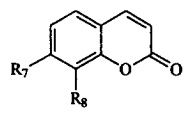
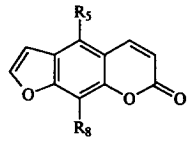
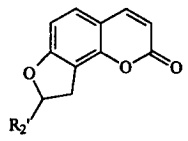


	R_7	R_8	
1	H	H	coumarin
2	OH	H	umbelliferone
8	OCH_3	CH_2-CH=C$(CH_3)_2$	osthol



	R_5	R_8	
4	H	H	psoralen
5	H	OCH_3	xanthotoxin
6	OCH_3	H	bergapten



	R_2	
3	C$(OH)(CH_3)_2$	columbianetin
7	C$(O$-CO-$CH_3)(CH_3)_2$	columbianetin acetate
9	C$(O$-CO-C(CH_3)=CH$CH_3)(CH_3)_2$	columbianadin

Fig. 1. Structures of the coumarins studied.

psoralen, xanthotoxin and bergapten were purchased from Aldrich (Milwaukee, WI, USA), 4-hydroxybenzoic acid from Merck (Darmstadt, Germany), sodium dodecyl sulfate (SDS) from Sigma (St. Louis, MO, USA), sodium borate from Nakarai Chemicals (Kyoto, Japan) and sodium dihydrogenphosphate from Kanto (Tokyo, Japan). Angelicae Tuhou Radix was obtained from the Chinese herbal market in Taipei (Taiwan). Acetonitrile and methanol of LC grade were obtained from Mallinckrodt (Paris, KY, USA). Deionized water was provided by a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

2.2. Apparatus and conditions

MEKC analysis was carried out on a Spectra Phoresis 1000 capillary electrophoresis system (Spectra-Physics, San Jose, CA, USA) equipped with a UV detector set at 200 nm and a 67.5 cm × 50 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) with the detection window placed at 59.5 cm. The conditions were as follows: injection mode, hydrodynamic; injection time, 2 s; run time, 25 min; applied voltage, 20 kV (constant voltage, positive to negative polarity); cartridge temperature, 30°C. The electrolyte was buffer solution [20 m*M* sodium dodecyl sulfate (SDS)–15 m*M* sodium borate–15 m*M* sodium dihydrogenphosphate (pH 8.26)]–acetonitrile (24:1). The capillary was washed with 0.1 *M* NaOH for 3 min, deionized water for 3 min and buffer for 3 min before each run and washed with deionized water for 3 min after each run.

HPLC analysis was performed on a Waters (Milford, MA, USA) HPLC system consisting of two Model 510 pumps, a U6K injector, a Model 680 automated gradient controller and a Model 990 photodiode-array detector set at 322 nm. Satisfactory separation of the coumarins was achieved on a Cosmosil $5C_{18}$-AR column (250 mm × 4.6 mm I.D., particle size 5 μm) (Nacalai Tesque), which was eluted with a linear gradient of solvent A [H_2O–CH_3CN (8:2)] and solvent B [H_2O–CH_3CN–CH_3OH (2:9:9)] according to the following profile: 0–40 min, 100–0% A (0–100% B) and 40–50 min, 100% B. The flow-rate was maintained at 0.6 ml/min.

2.3. Preparation of Angelicae Tuhou Radix extract

A 1.00 g sample of powdered Angelicae Tuhou Radix was extracted with 70% methanol (5 ml) by refluxing for 10 min, then centrifuged at 1500 *g* for 5 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter-paper. After the addition of 2.00 ml of internal standard solution (2 mg of 4-hydroxybenzoic acid in 1 ml of 70% methanol), the Angelicae Tuhou Radix extract was diluted to 25 ml with 70% methanol.

3. Results and discussion

3.1. HPLC method

It is very common to assay coumarins by HPLC. Thompson and Brown [22] used seven

solvent systems in sequence to separate a total of 67 coumarins [22] and Vande Casteele et al. [23] utilized a combination of isocratic and linear-gradient elution to separate 43 coumarins. However, the previous HPLC methods could not be easily applied to the simultaneously separation of the nine coumarins selected.

A preliminary experiment was first conducted with isocratic elution using 60% and 50% acetonitrile at a flow-rate of 0.6 ml/min. In both instances, the nine compounds gave only eight peaks, compounds **4** and **5** being completely overlapped. At 60% acetonitrile, the analysis could be accomplished within 20 min but the peaks of **2** and **3** were too close together. With 50% acetonitrile, a good separation of **1–3** and **6–9** could be obtained; the elution time was 42 min and peaks **8** and **9** were broad. After a series of experiments, it was found that linear-gradient elution with the profile given in Section 2.2 separated all the coumarins well. Fig. 2a shows the separation of the authentic coumarins with the following retention times: **2**, 17.8; **1**, 25.1; **3**, 25.7; **4**, 30.9; **5**, 31.5; **6**, 35.0; **7**, 37.5; **8**, 45.1 and **9**, 46.0 min. It is noticeable that a marked change in retention times was obtained with only slight variations of mobile phase composition. When the water–acetonitrile component of solvent B was changed to 1:9 and solvent A kept constant, the retention times of the coumarins were as follows: **2**, 16.8; **3**, 23.5; **1**, 23.7; **4**, 28.2; **5**, 28.6; **6**, 31.2; **7**, 33.4; **8**, 40.3; and **9**, 41.5 min. Thus not only was a much worse resolution of **4** and **5** obtained, but also a completely overlapped peak for **1** and **3** was found.

3.2. MEKC method

All nine coumarins and 4-hydroxybenzoic acid (internal standard) were successfully determined in a single run by MEKC under suitable conditions. The separation was achieved by optimizing the cartridge temperature, the applied voltage and the concentrations of SDS, organic modifier and buffer.

Cartridge temperatures of 20, 25, 28, 30, 35, 40 and 45°C and applied voltages of 15, 18, 20, 22, 25 and 28 kV were studied. Values of 30°C and 20 kV were chosen, because longer migration times and broader peak widths were ob-

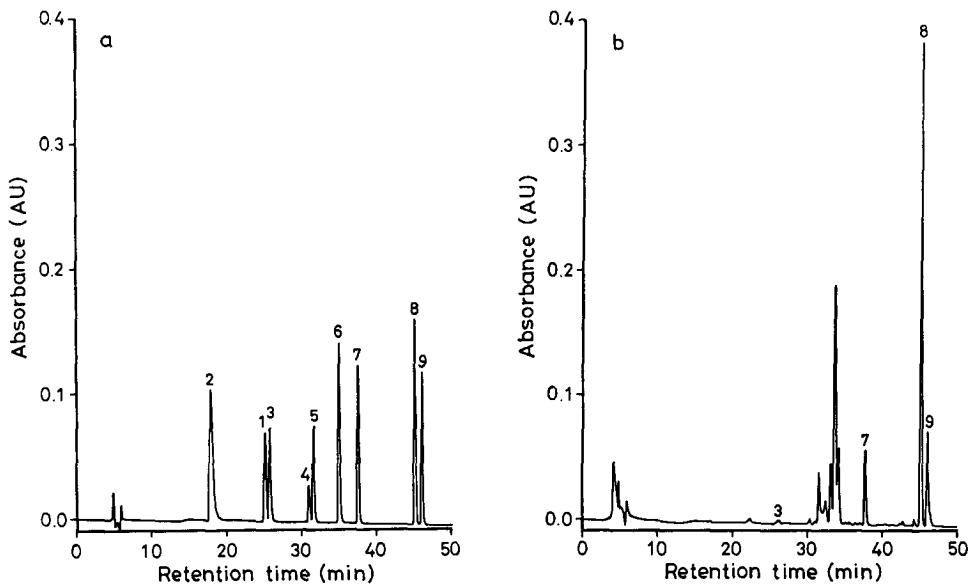


Fig. 2. HPLC of (a) a mixture of nine authentic coumarins and (b) an extract of Angelicae Tuhou Radix. Column: Cosmosil $5C_{18}$-AR (250 mm × 4.6 mm I.D., particle size 5 μm). Eluents: solvent A, H_2O–CH_3CN (8:2); solvent B, H_2O–CH_3CN–CH_3OH (2:9:9). Elution profile: 0–40 min, 100–0% A (0–100% B); 40–50 min, 100% B. Detection: UV at 322 nm. Peak numbers as in Fig. 1.

tained with lower values and a poorer resolution for the test solution was found with higher values.

Preliminary experiments were first carried out using 15 m*M* $Na_2B_4O_7$ and 15 m*M* NaH_2PO_4 without SDS in the electrophoretic medium. In this case, all courmarins except umbelliferone migrated with the electroosmotic flow (EOF), indicating that all the eight coumarins possess neutral and similar properties under such conditions. However, with SDS, the components in the mixture sample can be separated on the basis of their relative affinities for the micellar environment against the bulk aqueous phase. In order to study the effect of SDS concentration on the separability, seven electrolyte systems containing different SDS concentrations (0, 5, 10, 20, 30, 40 and 50 m*M*) were used. The results obtained are shown in Fig. 3. The migration times of all the compounds became longer as the SDS concentration increased. At 20 m*M* SDS, most peaks were separated very well except for **3** and **4**, which slightly overlapped. At lower concentrations (5 or 10 m*M*), **3** and **4** completely overlapped. At higher concentrations (30, 40 or 50 m*M*), the resolution between **8** and **9** became poorer. The resolution values (R_s) between **8** and **9** were 1.54, 0.67, 0.51 and 0.37 when 20, 30, 40 and 50 m*M* SDS were used, respectively.

It is known that the addition of organic modifier to the buffer solution can greatly improve

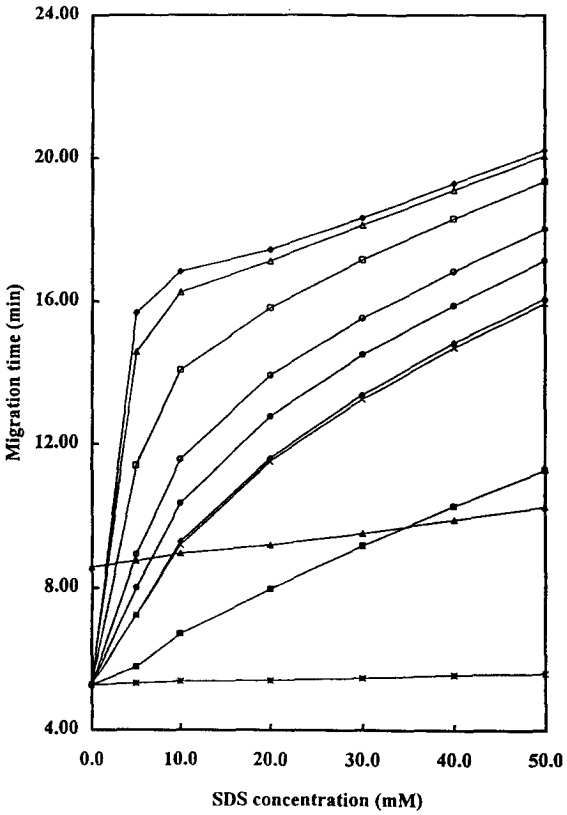


Fig. 3. Effect of SDS concentration on migration time. Electrophoretic medium: 0–50 m*M* SDS–15 m*M* $Na_2B_4O_7$–15 m*M* NaH_2PO_4. Capillary: 67.5 cm (59.5 cm to detector) × 50 μm I.D. Applied voltage: 20 kV. Cartridge temperature: 30°C. Detection: UV at 200 nm. * = EOF; ■ = **1**; ▲ = **2**; ◆ = **3**; × = **4**; ● = **5**; ○ = **6**; □ = **7**; △ = **8**; ◇ = **9**.

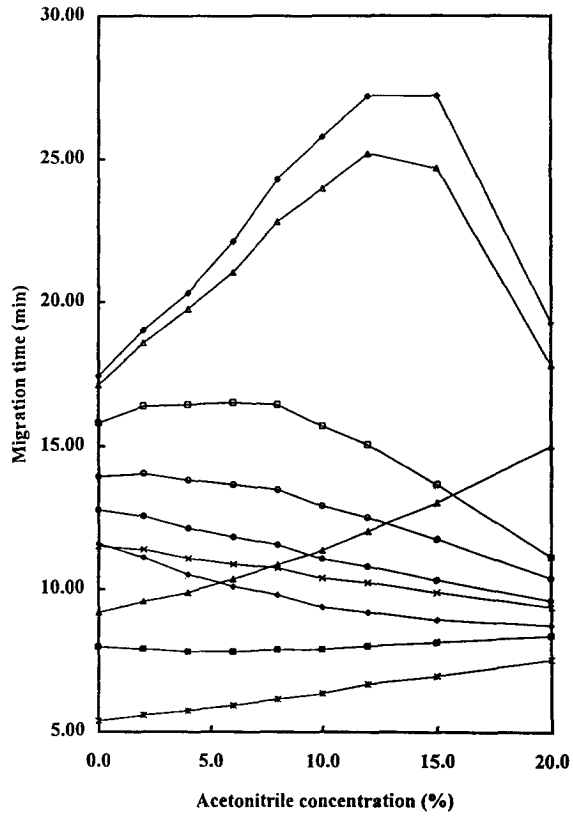


Fig. 4. Effect of CH_3CN concentration on migration time. Electrophoretic medium: mixture of 0–20% CH_3CN with a buffer of 20 m*M* SDS–15 m*M* $Na_2B_4O_7$–15 m*M* NaH_2PO_4. Other conditions and symbols as in Fig. 3.

the resolution of the solutes. Fig. 4 shows the effect of acetonitrile concentration (0–20%) on the selectivity of the separation. The electroosmotic flow decreases with increasing acetonitrile concentration. The migration times would be longer when the factor causing the decrease in electroosmotic flow was dominant. On the other hand, the migration times would become shorter if the factor lessening the interaction between solute and micelles was dominant. Fig. 4 indicates that the migration times of **2**, **8** and **9** became longer and those of **3–6** became shorter as the acetonitrile concentration was increased from 0 to 12%. At 20% acetonitrile, the migration times of most compounds became much shorter owing to the latter factor being completely dominant. In addition, the resolution between **3** and **4** was obviously improved when acetonitrile was added. The R_s values between **3** and **4** were 1.54, 2.10 and 3.42 when 0, 2 and 4% of acetonitrile were used, respectively. At 4% acetonitrile, **3** and **4** could be completely separated.

The buffer concentration and the pH of the electrophoretic medium are two other governing factors in separation. After a series of experiments, it was found that a solution consisting of 15 m*M* $Na_2B_4O_7$–15 m*M* NaH_2PO_4 (pH 8.26) was the optimum, and an electrolyte consisting of buffer solution (20 m*M* SDS–15 m*M* $Na_2B_4O_7$–15 m*M* NaH_2PO_4)–acetonitrile (24:1) was found to give the best resolution. Fig. 5a is an electropherogram showing the separation of the nine coumarins with the following migration times and plate numbers: **1**, 7.5 min, $3.04 \cdot 10^4$; **2**, 9.6 min, $2.76 \cdot 10^5$; **3**, 10.4 min, $1.10 \cdot 10^5$; **4**, 10.9 min, $9.96 \cdot 10^4$; I.S., 11.4 min, $8.61 \cdot 10^4$; **5**, 12.1 min, $1.08 \cdot 10^5$; **6**, 13.9 min, $9.52 \cdot 10^4$; **7**, 16.8 min, $1.10 \cdot 10^5$; **8**, 20.3 min, $4.42 \cdot 10^4$; and **9**, 21.0 min, $1.02 \cdot 10^5$.

3.3. Method validation

The λ_{max} of most coumarins is around 200 nm, which can be used as the detection wavelength for MEKC measurements but not for HPLC owing to a serious cut-off from the eluent in this region. A wavelength of 322 nm, another absorption maximum for **2**, **3**, **7**, **8** and **9** in UV

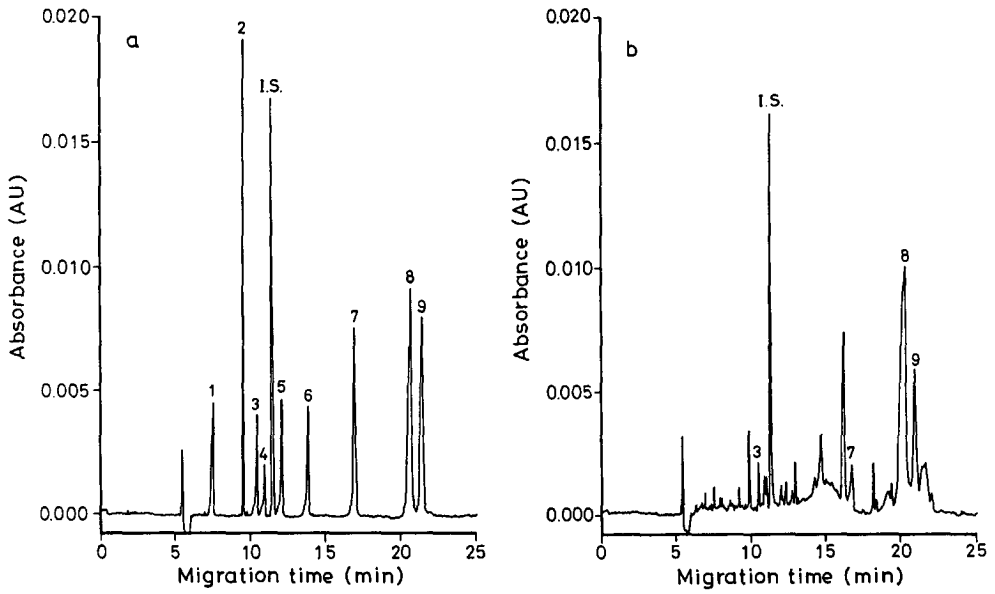


Fig. 5. Capillary electropherograms of (a) a mixture of nine authentic coumarins and (b) an extract of Angelicae Tuhou Radix. Electrophoretic medium: buffer (20 m*M* SDS–15 m*M* $Na_2B_4O_7$–15 m*M* NaH_2PO_4)–CH_3CN (96:4). Capillary: 67.5 cm (59.5 cm to detector) × 50 μm I.D. Applied voltage: 20 kV. Cartridge temperature: 30°C. Detection: UV at 200 nm. Peak numbers as in Fig. 1; I.S. (internal standard) = 4-hydroxybenzoic acid.

spectra, was therefore chosen for the HPLC assay. The sensitivities at 200 nm are about 1.16–3.57 times those of 322 nm. The detection limits (signal-to-noise ratio = 3) of these coumarins in HPLC varied from 0.20 to 0.38 μg/ml (flow cell length 10 mm) and those in MEKC are listed in Table 1.

Linearity

Calibration graphs (peak-area ratio, y, vs. concentration in mg/ml, x) were constructed in the range $9.28 \cdot 10^{-3}$–0.186 mg/ml for **3**, $5.60 \cdot 10^{-3}$–0.112 mg/ml for **4** and $1.60 \cdot 10^{-2}$–0.320 mg/ml for other seven compounds. Linear relationships were found with the correlation coefficients >0.999.

Precision

The reproducibility (R.S.D.) of the MEKC method, on the basis of peak-area ratios for six replicate injections, is shown in Table 1. The R.S.D.s of the migration time of each peak for six replicated injections were below 0.65% (intra-day) and 1.35% (inter-day). The R.S.D.s for the HPLC method, on the basis of peak area for six replicate injections, were 0.52–1.76% (intra-day) and 0.63–2.31% (inter-day) and the R.S.D.s of the retention time for six replicated injections were below 0.18% (intra-day) and 0.33% (inter-day).

Table 1
Detection limits and reproducibility of separation of coumarins.

Coumarin	Detection limit (μg/ml)	R.S.D. (%) ($n = 6$)	
		Intra-day	Inter-day
1	3.93	1.05	1.44
2	1.60	0.61	0.90
3	4.08	1.42	1.45
4	2.80	1.86	2.43
5	3.82	1.31	2.00
6	3.67	1.27	1.40
7	2.29	1.79	2.59
8	2.57	2.37	3.94
9	3.89	0.77	1.89

Accuracy

Suitable amounts of the nine coumarins were added to a standard solution with known concentrations of components and analysed using the proposed procedure. The recoveries of all coumarins determined were around 98–103%.

The asymmetry factor was 0.81 for **1**, 1.00 for **2**, 0.79 for **3**, 0.82 for **4**, 1.00 for I.S., 0.88 for **5**, 0.88 for **6**, 0.90 for **7**, 0.91 for **8** and 0.94 for **9**.

3.4. Separation of coumarins in Angelicae Tuhou Radix

When the test solution was analyzed by MEKC under the selected conditions, the electropherogram shown in Fig. 5b was obtained. The peaks were identified by comparison of the migration times and UV spectra with those obtained from authentic samples of the coumarins, and by spiking the mixture with a single coumarin in a subsequent run. It was found that only columbianetin, columbianetin acetate, osthol and columbianadin were present in the extract of Angelicae Tuhou Radix. By substituting the peak-area ratios of the individual peaks for y in the above equations, the contents of the individual coumarins in the test sample were obtained: **3**, 0.36 ± 0.03; **7**, 0.60 ± 0.03; **8**, 3.89 ± 0.20; and **9**, 1.88 ± 0.20 mg/g (mean ± S.D.; $n = 4$). Amounts of 0.25 mg of **3**, 0.24 mg of **7**, 0.97 mg of **8** and 0.56 mg of **9** were added to a sample of Angelicae Tuhou Radix with known concentrations of components and analysed using the proposed procedures. The recoveries of these coumarins determined were around 97–110%.

The test solution was also analysed by HPLC and the chromatogram is shown in Fig. 2b. A smoother baseline and much better resolution of **8** and **9** than those obtained by MEKC were obtained, but no separated peaks were observed in the 30–35-min region. Therefore, it is very difficult to ascertain whether or not **4** and **5** were present in the crude extract using the HPLC method.

In conclusion, the coumarins in crude drug extracts could be successfully determined either by MEKC or HPLC. The MEKC method gives a

shorter run time (MEKC, 22 min; HPLC, 47 min) and can provide better resolution for **4** and **5**, if present, but shows worse baseline noise and gives a lower resolution for **8** and **9**.

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References

[1] R.D.H. Murray, J. Méndez and S.A. Brown, The Natural Coumarins, Wiley, Chichester, 1982.
[2] H.Y. Hsu, Y.P. Chen, S.J. Sheu, C.H. Hsu, C.C. Chen and H.C. Chang, Chinese Materia Medica—A Concise Guide, Modern Drug Press, Taipei, 1985, pp. 244–245.
[3] K. Hata and Y. Tanaka, Yakugaku Zasshi, 77 (1957) 937.
[4] Y. Tanaka, Kinki Daigaku Yakugakubu Kiyo, 4 (1963) 17; C.A., 59 (1963) 14354h.
[5] K. Hata and M. Kozawa, Tetrahedron Lett., 50 (1965) 4557.
[6] K. Hata, M. Kozawa, K. Baba and K.Y. Yen, Chem. Pharm. Bull., 19 (1971) 640.
[7] K. Hata and M. Kozawa, Yakugaku Zasshi, 87 (1967) 210.
[8] K. Hata and M. Kozawa, Yakugaku Zasshi, 88 (1968) 283.
[9] K. Hata and M. Kozawa, Yakugaku Zasshi, 88 (1968) 293.
[10] M. Kozawa, K. Baba, Y. Matsuyama and K. Hata, Chem. Pharm. Bull., 28 (1980) 1782.
[11] K. Hata, T. Nishino, Y. Hirai, Y. Wada and M. Kozawa, Yakugaku Zasshi, 101 (1981) 67.
[12] K. Baba, Y. Matsuyama and M. Kozawa, Chem. Pharm. Bull., 30 (1982) 2025.
[13] S. Chen, H. Zhang, C. Yuan, Y. Deng and G. Chen, Yaoxue Tongbao, 17 (1982) 120; C.A., 97 (1982) 20741y.
[14] S. Chen, H. Zhang, C. Yuan, Y. Deng and G. Chen, Yaoxue Tongbao, 17 (1982) 392; C.A., 97 (1982) 88731a.
[15] J. Pan, K. Lamy, B. Arison, J. Smith and G. Han, Yaoxue Tongbao, 22 (1987) 380; C.A., 107 (1987) 112685m.
[16] X. Li, W. Fang and R. Li, Zhongcaoyao, 21 (1990) 59; C.A., 112 (1990) 185907y.
[17] Z.F. Sha, W.J. Sun, H. Gao and M.L. Miao, Yaoxue Xuebao, 26 (1991) 798; C.A., 116 (1992) 136345n.
[18] H. Gao, W.J. Sun and Z.F. Sha, Yaowu Fenxi Zazhi, 12 (1992) 221; C.A., 117 (1992) 220206w.
[19] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem., 56 (1984) 111.
[20] Y.M. Liu and S.J. Sheu, Anal. Chim. Acta, 288 (1994) 221.
[21] C.F. Lu and S.J. Sheu, Chin. Pharm. J., 45 (1993) 495.
[22] H.J. Thompson and S.A. Brown, J. Chromatogr., 314 (1984) 323.
[23] K. Vande Casteele, H. Geiger and C.F. Van Sumere, J. Chromatogr., 258 (1983) 111.