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Note

Determination of coumarins in *Cnidium monnieri fructus* by high-performance liquid chromatography

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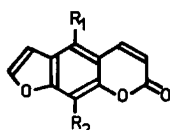
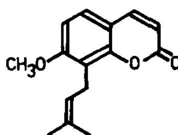
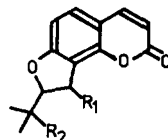
The Chinese crude drug *Cnidium monnieri fructus* (She chuang zi) has been used as a tonic and for the treatment of skin disease[1]. A number of coumarins, such as xanthotoxin[2], isopimpinerin[2–4], bergapten[2–6] and imperatorin[2,3] (which are linear-type furocoumarins), columbianadin[2,3], edultin[2–4] and columbianetin[2–4] (which are angular-type dihydrofurocoumarins) and osthol[2,3], are contained in this crude drug. In recent years, it has also been reported that its antidermatophytic activity[5], antiallergic activity[6] and calcium channel antagonistic activity[7] are derived from the coumarins, mainly osthol. The origin of *Cnidium monnieri fructus* derives from two plants, *Cnidium monnieri* and *Cnidium formosanum*, and it is reported that the former contains osthol as the main component and has no angular-type dihydrofurocoumarins, whereas the latter does not contain osthol[2,5]. Therefore, it is important for the evaluation of this crude drug to analyse their coumarin constituents.



I Linear type furocoumarins



II



III Angular type dihydrofurocoumarins

	R1	R2
Xanthotoxin (XA)	H	OCH_3
Isopimpinerin (IS)	OCH_3	OCH_3
Bergapten (BE)	OCH_3	H
Alloimperatorin (AL)	[prenyl]	OH
Imperatorin (IM)	H	O–[prenyl]

Osthol (OS)

	R1	R2
Columbianadin (COD)	H	OOC–[structure]
Columbianetin (COT)	H	OH
3'-Isobutyryloxy-O-acetylcolumbianetin (IAC)	OOC–[structure]	OAc
Edultin (ED)	OOC–[structure]	OAc
O-Acetylcolumbianetin (AC)	H	OAc

Thin-layer chromatography (TLC) has been used in the evaluation of *Cnidium monnieri fructus*[5], but the coumarin content has not been completely resolved by this method. For the separation of coumarins, several high-performance liquid chromatographic (HPLC) methods, using either reversed-phase or normal-phase columns, have been reported[8–17]. Thompson and Brown[14] reported the behavior of 67

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kinds of coumarins on a normal-phase silica column and a C_{18} reversed-phase column and applied the normal-phase column to the resolution of coumarins in *Citrus aurantifolia.*

The aim of this study was to demonstrate the application of an HPLC method to the separation and determination of coumarins in *Cnidium monnieri fructus* and to determine the difference in coumarin contents in *Cnidium monnieri* and *Cnidium formosanum*, the source of *Cnidium monnieri.*

EXPERIMENTAL

Plant materials

Commercial *Cnidium monnieri fructus* samples were purchased from Matsuura Yakugyo and Kinokuniya Kan Yakkyoku.

Apparatus

A Hitachi Model 655 liquid chromatograph equipped with a Uvilog 5IV UV spectrophotometer and a stainless-steel column (150 × 4 mm I.D.) packed with chemically bonded ODS silica gel (TSK gel LS-410, 5 μm; Toyo Soda) or a stainless-steel column (250 × 4 mm I.D.) packed with silica gel (LiChrosorb Si 60, 5 μm; Merck) was used. Silica gel plates (Kieselgel 60 F_{254}, 0.25 mm layer; Merck) were used for TLC.

Reagents

Xanthotoxin, isopimpinerin, bergapten, alloimperatorin, osthol, columbianadin, 3′-isobutyryloxy-O-acetylcolumbianetin, edultin and O-acetylcolumbianetin were isolated from *Cnidium monnieri fructus,* purified by HPLC and recrystallized. Columbianetin was derived from columbianadin by hydrolysis. The acetonitrile, *n*-hexane and ethyl acetate used for the chromatography and ethyl salicylate were of special grade (Tokyo Kasei's guaranteed reagent). Ion-exchanged water was further purified using a Millipore filter.

HPLC conditions

Mixtures of water–acetonitrile (65:35) for reversed-phase HPLC and *n*-hexane–ethyl acetate (8:2) for normal-phase HPLC were used as the mobile phase. The temperature of the reversed-phase column was maintained at 50°C and the normal-phase column was kept at room temperature. The flow-rate was 1.0 ml/min. The substances eluted were detected by a UV detector operated at 320 nm.

Assay procedure

Cnidium monnieri fructus was pulverized, placed in 15 ml of 50% acetonitrile and heated under reflux on a water-bath at 85°C. After cooling, it was centrifuged at 1660 *g* and decanted. The residue was extracted twice by the same method. All extracts were placed in a 50-ml volumetric flask and diluted to 50 ml with 50% acetonitrile. Portions of 5 ml of this extract, accurately measured, were placed in a 10-ml volumetric flask, 2.5 ml of internal standard solution (2.5% ethyl salicylate in ethanol) were added and the mixture was diluted to 10 ml with 50% acetonitrile. A 10-μl volume of this solution was injected for HPLC. The content of each coumarin

in the samples was calculated from the ratio of the peak area to the internal standard peak area.

Calibration graphs and detection limits

Calibration graphs for xanthotoxin, isopimpinerin, bergapten, O-acetylcolumbianetin, imperatorin, osthol, 3′-isobutylyroxy-O-acetylcolumbianetin, edultin and columbianadin were obtained for the concentration ranges 2.0–20.0, 2.0–20.0, 2.0–20.0, 2.0–20.0, 5.0–50.0, 10.0–200.0, 8.0–80.0, 8.0–80.0 and 8.0–80.0 μg/ml, respectively the corresponding regression equations were as follows: $y = 9.63x - 0.41$ ($r = 0.999$); $y = 9.47x + 0.09$ ($r = 0.999$); $y = 12.1x + 0.3$ ($r = 0.999$); $y = 18.0x - 0.7$ ($r = 0.999$); $y = 7.51x + 0.29$ ($r = 0.999$); $y = 10.2x - 1.3$ ($r = 0.999$); $y = 12.1x + 1.1$ ($r = 0.999$); $y = 10.6x - 1.5$ ($r = 0.999$) and $y = 12.7x + 0.0$ ($r = 0.999$), respectively. The detection limits were 0.2, 0.2, 0.2,0.3,2.0, 0.5, 1.0, 1.0 and 1.0 ng, respectively, at a signal-to-noise ratio of 3:1 for the peak heights.

RESULTS AND DISCUSSION

Separation parameters such as the kind and concentration of organic modifier, addition of certain salts and variation of pH and column temperature were examined to establish the optimum conditions for the separation of coumarins in the crude drug *Cnidium monnieri fructus* by HPLC using an ODS column.

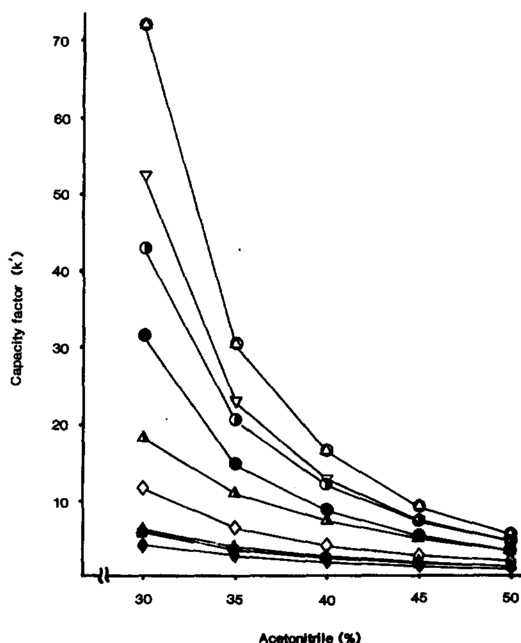

Fig. 1. Effect of acetonitrile concentration on the capacity factors, k', of xanthotoxin (◆), isopimpinerin (■), bergapten (▲), O-acetylcolumbianetin (◇), ethyl salicylate (internal standard) (▲), imperatorin (●), osthol (◑), 3′-isobutyryloxy-O-acetylcolumbianetin (▽), edultin (○) and columbianadin (△). Closed symbols represent linear-type furocoumarins and open symbols angular-type dihydrofurocoumarins. Mobile phase, water–acetonitrile; flow-rate, 1 ml/min; temperature, 50°C.

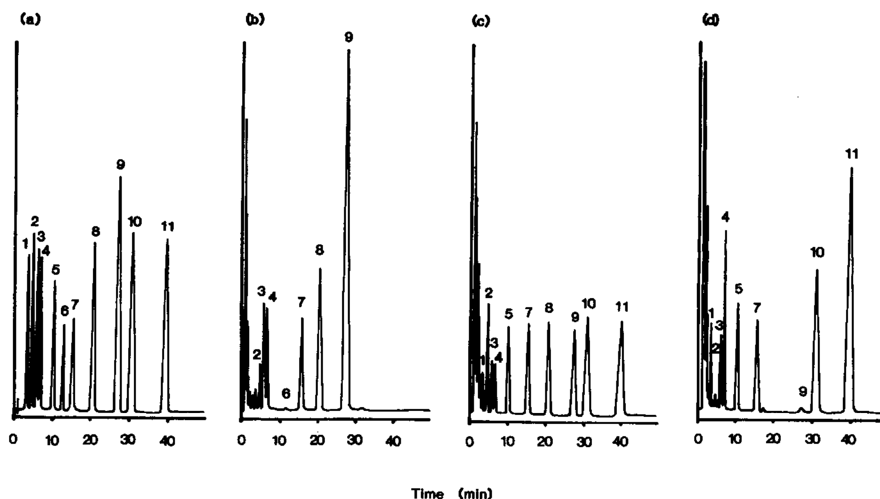

Fig. 2. Chromatograms of standards and samples. (a) Standards; (b) sample 1 (China, Shandong); (c) sample 9 (China, Beijing); (d) sample 11 (market, Tokyo). Peaks: 1 = columbianetin; 2 = xanthotoxin; 3 = isopimpinerin; 4 = bergapten; 5 = O-acetylcolumbianetin; 6 = alloimperation; 7 = ethyl salicylate (internal standard); 8 = imperatorin; 9 = osthol; 10 = 3'-isobutyryloxy-O-acetylcolumbianetin; 11 = edultin + columbianadin.

The addition of some salts to the mobile phase and variation of the pH of the mobile phase did not affect the capacity factors of the neutral coumarins. Fig. 1 shows the effect of acetonitrile concentration in the mobile phase on the capacity factors. The optimum composition of the mobile phase, with regard to both the separation of coumarins and the analysis time, was water–acetonitrile (65:35). Edultin

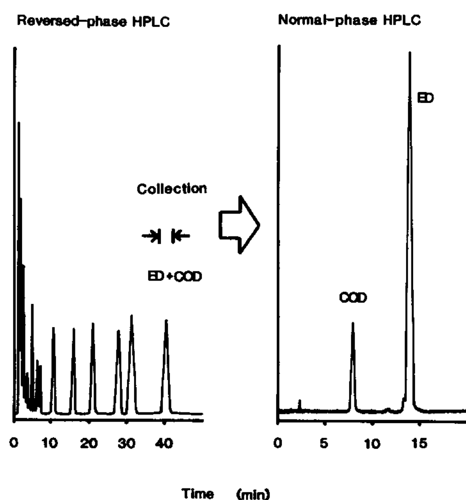

Fig. 3. Chromatogram of columbianadin and edultin on a normal-phase column. Peaks: ED = edultin; COD = columbianadin. Column: LiChrosorb Si 60 (5 μm), 25 cm × 4 mm I.D. Mobile phase, *n*-hexane–ethyl acetate (8:2); flow-rate, 1 ml/min; temperature, room temperature.

and columbianadin, which are angular-type dihydrofurocoumarins, were not resolved with this HPLC system even when each separation parameter was varied. Ethyl salicylate, the internal standard, was eluted between alloimperatorin and imperatorin. The chromatograms of all coumarin standards and samples are shown in Fig. 2. As edultin and columbianadin were not separated, a silica gel column was used for their resolution and determination. After collecting the peak eluted under reversed-phase conditions, the collected fraction was re-analysed on a silica gel column with *n*-hexane–ethyl acetate (8:2) as the mobile phase (Fig. 3). However, using this normal-phase column the other compounds were not separated completely.

Since the method for the extraction of coumarins from *Cnidium monnieri fructus* had not previously been examined in detail, several solvents were tried for their extraction. *n*-Hexane, which is non-polar solvent, could better extract aprotic coumarins such as 3′-isobutyryloxy-O-acetylcolumbianetin than protic coumarins such as xanthotoxin. Conversely, when a polar solvent such as water was used, the efficiency of extraction of polar coumarins was better than that of non-polar coumarins. Ethanol and acetonitrile were each better solvents than chloroform, which is usually used for the extraction of coumarins from the plant. Further, acetonitrile–water showed good extraction efficiency, a 1:1 mixture being optimum (Table I).

The HPLC method established from this examination was compared with the TLC method[5]. Using the TLC method, poor resolutions between bergapten, O-acetylcolumbianetin and imperatorin and between xanthotoxin, isopimpinerin, 3′-isobutyryloxy-O-acetylcolumbianetin and edultin were obtained (Fig. 4). Therefore, TLC cannot be used for their quantitative determination. On the other hand, the HPLC method was able to separate, at least in this experiment, nine kinds of coumarins, except columbianadin and edultin, and to determine seven components. Columbianetin and alloimperatorin were also detected, but they were not determined owing to the existence of an impurity in columbianetin and a trace amount of alloimperatorin in this crude drug. This is the first time that alloimperatorin has been detected in *Cnidium monnieri fructus*.

Cnidium monnieri fructus originates from two plants, *Cnidium monnieri* and

TABLE I

EFFECT OF SOLVENT ON EXTRACTION EFFICIENCY (%)

For compounds, see formulae in the Introduction.

Solvent	*I**				*OS*	*II***	
	XA	*IS*	*BE*	*IM*		*AC*	*IAC*
Acetonitrile–water (1:1)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
n-Hexane	45.7	58.8	49.5	74.2	66.3	60.1	83.8
Chloroform	77.9	83.3	76.5	88.7	80.4	70.9	86.4
Ethyl acetate	68.6	78.6	70.7	83.5	78.5	67.7	81.7
Ethanol	91.0	95.2	90.4	98.1	90.6	78.8	93.7
Acetonitrile	85.3	91.8	85.2	93.9	87.5	73.6	87.4
Water	45.7	34.9	27.3	6.1	5.1	21.2	7.1

* Linear-type furocoumarins.

** Angular-type dihydrofurocoumarins.

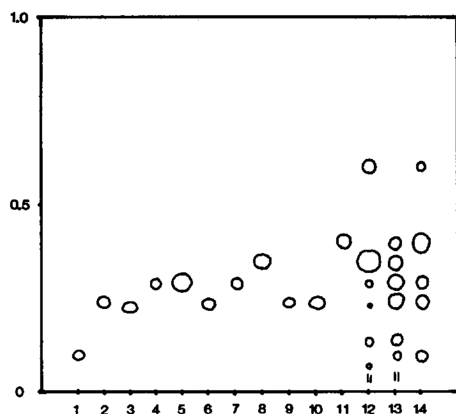

Fig. 4. TLC pattern of *Cnidium monnieri fructus*. TLC plate: Kieselgel 60 F_{254}. Development solvent : *n*-hexane–ethyl acetate (3:1), twice. 1 = Columbianetin; 2 = xanthotoxin; 3 = isopimpinerin; 4 = bergapten; 5 = O-acetylcolumbianetin; 6 = alloimperatorin; 7 = imperatorin; 8 = osthol; 9 = 3′-isobutyryloxy-O-acetylcolumbianetin; 10 = edultin; 11 = columbianadin; 12 = sample 1 (China, Shandong); 13 = sample 9 (China, Beijing); 14 = sample 11 (market, Tokyo).

Cnidium formosanum, which can be distinguished by their coumarin constituents. Baba and co-workers[2,5] reported that *Cnidium monnieri* contains osthol as the main component, with no angular-type dihydrofurocoumarins, whereas the main components of *Cnidium formosanum* are angular-type dihydrofurocoumarins. We re-examined these plants by HPLC and did not detect any significant angular-type dihydrofurocoumarins in samples 1–6. Osthol was not detected in the sample purchased in Tokyo (Table II). From these results, it is suggested that the first six samples

TABLE II

COUMARIN CONTENTS (%) IN *CNIDIUM MONNIERI FRUCTUS* SAMPLES

For compounds, see formulae in the Introduction.

Sample No.	*Source*	*I**				*OS*	*II***		
		XA	*IS*	*BE*	*IM*		*AC*	*IAC*	*ED+COD****
1	China, Shandong	0.07	0.17	0.14	0.89	2.32	Trace	0.01	–
2	China, Shanghai	0.13	0.16	0.11	0.83	2.43	Trace	0.01	–
3	China, Hubei	0.12	0.13	0.11	0.87	2.21	Trace	0.01	Trace
4	Market, Hongkong	0.19	0.13	0.12	1.03	2.65	Trace	0.01	–
5	Market, Nagoya	0.16	0.12	0.10	0.88	2.30	Trace	0.01	–
6	Market, Nagoya	0.19	0.13	0.11	0.93	2.44	Trace	Trace	–
7	China, Huanan	0.15	0.11	0.07	0.67	1.44	0.04	0.14	+
8	Market, Nagoya	0.13	0.10	0.07	0.63	1.46	0.03	0.11	+
9	China, Beijing	0.16	0.09	0.10	0.59	0.64	0.11	0.31	+ +
10	Market, Beijing	0.17	0.09	0.08	0.57	0.33	0.11	0.38	+ + +
11	Market, Tokyo	Trace	0.08	0.18	–	0.03	0.07	0.21	+ + +

* Linear-type furocoumarins.
** Angular-type dihydrofurocodumarins.
*** +, <1.0%; + +, 1.0–2.0%; + + +, >2.0% as columbianadin content.

originated from *Cnidium monnieri* and the source of the Tokyo sample is *Cnidium formosanum*. Although it has been said that an intermediate type of component does not exist in the originating plant of *Cnidium monnieri fructus*, some intermediate-type crude drugs were found. Linear-type furocoumarins were present in all samples except imperatorin from the sample purchased in Tokyo. Columbianadin and osthol could be detected by TLC, but the existence of other coumarins was not obvious. In contrast, using HPLC, many coumarins could be detected and determined. HPLC therefore permitted a detail evaluation of *Cnidium monnieri fructus*. There is some doubt whether an intermediate type exists or whether this is due to a mixture of *Cnidium monnieri* and *Cnidium formosanum*, because the osthol content is roughly inversely proportional to the angular-type dihydrofurocoumarin. This question may be resolved after cultivation studies and further analysis by HPLC.

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