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Isolation and Gas Chromatographic Method for Determination of Osthole from Cnidii Fructus

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ABSTRACT

The purpose of this research is to study the isolation and purification of osthole from Cnidii Fructus extract and to establish a gas chromatographic method for determining the osthole content from various Cnidii Fructus samples. Six solvents were used to extract Cnidii Fructus in order to evaluate their efficiency in extracting osthole. Vacuum fractional distillation and traditional column chromatography were compared to evaluate the efficiency in purifying osthole from Cnidii Fructus extract. Gas chromatographic method was used to determine osthole content in Cnidii Fructus extract. The analytical conditions are the following: nitrogen as the carrier gas, the flow rate of 40 mL/min; the split ratio of 120 : 1. The column used was DB^{TM}-5 (30 m × 0.53 mm I.D., 1.5 µm) equipped with a flame ionization detector (FID). The initial oven temperature was programmed to be at 135˚C for 12 minutes. The temperature was then raised to 215˚C at a rate of 12˚C /min for 20 minutes. The retention time of osthole was 24.1 min. Caffeine anhydrous was used as the internal standard with a retention time of 18.9 minutes. A comparative evaluation showed that aqueous alcoholic solvent was the most efficient solvent among six used in extraction of osthole from a Cnidii Fructus. Vacuum fractional distillation is useful for purifying osthole from Cnidii Fructus extract. It was found that the osthole content of commercial Cnidii Fructus samples varied from 0.05% to 2.14%. And the osthole content of Cnidii Fructus samples collected from Southern China was higher than those collected from Northern China.

Key words: Cnidii Fructus, osthole, gas chromatography

INTRODUCTION

Cnidii Fructus considered one of the topnotch Chinese medicines[1], was recorded for the first time. The fruit of *Cnidium monnieri* (L.) Cuss was identified as the origin of Cnidii Fructus confirmed by researches of Modern Chinese Herbal. The habitat of Cnidii Fructus includes Mainland China, Taiwan and Japan[2]. Connate Cnidii Fructus has different chemical composition in different producing areas. Linear type furanocoumarin is predominant in South China, while angular type dihydrofuranocoumarin preponderates in the north side. These two types differ in activity and toxicity[2,3]. As for Cnidii Fructus in Japan, the fruit of *Cnidium japonicum* Miq. is used[2]. Some medicinal materials such as *Daucus carota* L., *Torilis anthriscus* (Houtt.) DC., *Lappula echinata var.* heteracatha Gilib., *Carpesium abrotanoides* L.[2], were mistaken as Cnidii Fructus in the market.

Ancient books describe that Cnidii Fructus protects kidney, enhances sexuality, prevents cold and cures impotence, frigidness, pudendum eczema, female pruritus vulva, and trichomonad vaginitis[1]. On the other hand, current pharmacology study asserts that osthole prevents the secretion of hepatitis B virus[4], inhibits the proliferation of vascular smooth muscle cells[5] and relaxes trachea and pectoral aorta[6,7]. Osthole is also an effective ingredient to inhibit vaginal trichomonad. As a result, osthole content judges the quality of Cnidii Fructus[8]. In this study, osthole was used as a target ingredient, based on which, the specific gas chromatographic method was established. A fast and economical purification approach was developed according to the specific molecular characteristics of osthole.

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MATERIAL AND METHODS

I. *Material*

In this study, there were ten sets of Cnidii Fructus in total. The points of purchase included Taiwan, Japan, Southern China, Northern China and Inner Mongolia.

II. *Instrument and Gas chromatography*

The instrument used were: Mel-Temp melting point measurer (Laboratory Devices, US), Shimadzu IR-408 infrared spec (Shimadzu Corp., Japan), Hewlett Packard HP-8452A UV spec (HP, US), Varian Gemini –200 NMR (Varian Corp., US), Shimadzu GC14A GC chromatograph (Shimadzu Corp., Japan), FID spec, and Shimadzu CR6A integrator. Chromatographic condition is the following: Nitrogen gas as mobile phase, the flow rate of 40 mL/min, pre-column pressure of 1.3 kg/cm^2, diffluent ratio of 120:1, hydrogen gas pressure of 0.6 kg/cm^2, and air pressure is 0.5 kg/cm^2. The analytic column has the specification of DB^{TM}-5 ((5%-phenyl)-methylpolysiloxane, J&W Scientific), 30 m × 0.53 mm I.D. and 1.5 µm liquid film. One-pass temperature ascending program was applied. The initial oven temperature was programmed to be at 135˚C for

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12 minutes. The temperature was raised to 215˚C at a rate of 12˚C /min for 20 minutes. Temperature of injection inlet and FID was set at 180˚C. The volume of analytical sample was 1 µL. The vacuum pump of improved vacuum fractional distiller used was Shimadzu rotary oil vacuum pump SA-18 (Shimadzu Corp., Japan).

III. *Standard and Internal standard*

The standard of osthole was purchased from Yoneyama Corp. (Osaka, Japan). Internal standard was caffeine anhydrous, which was purchased from Pharmacopeial Convention, Inc. (US).

IV. *Experiment methods*

1. Separation, purification and identification of osthole

10 kg of Cnidii Fructus was extracted twice with 100 L of ethanol in heated reflux. Extracted liquid was filtered, concentrated, re-dissolved in hot petroleum ether, boiled and settled to be delaminated. The upper layer was concentrated into a paste form. 2.4 kg of condensate was obtained, from which 1.6 kg was taken for vacuum fractional distillation. 11.6 g of crude osthole was obtained at b.p.145-155˚C (0.03 mmHg). Separated osthole was recrystallized in diluted ethanol. 10 g of colorless acicular crystal was obtained and compared with standard. The relative purity was 100.02%. The remaining 0.8 kg of condensate was purified with column chromatography[9].

After analyzing ^{1}H-NMR, UV, IR and the documented data, the structure (Figure 1) was confirmed. The relative information was the following:

m.p. 83-84˚C[10,11]. b.p. 145-155˚C (0.03 mmHg). UV λ_{max} nm (log ε): 322 (3.9031), 258 (3.6335)[10]. IR ν cm^{-1}: 1718, 1605, 830[10,11]. ^{1}H-NMR (200 MHz, $CDCl_3$)δ : 1.66 (3H, s, J=0.4 Hz, H-15), 1.84 (3H, s, J=1.2 Hz, H-14), 3.53 (2H, d, J=7.2 Hz, H-11), 3.92 (3H, s, -OCH_3), 5.22 (1H, m, H-12), 6.23 (1H, d, J=9.2 Hz, H-3) : 6.83 (1H, d, J=8.6 Hz, H-6), 7.28 (1H, d, J=8.6 Hz, H-5), 7.62 (1H, d, J=9.2 Hz, H-4)[12,13].

2. Selection of the best solvent for osthole in Cnidii Fructus [14]

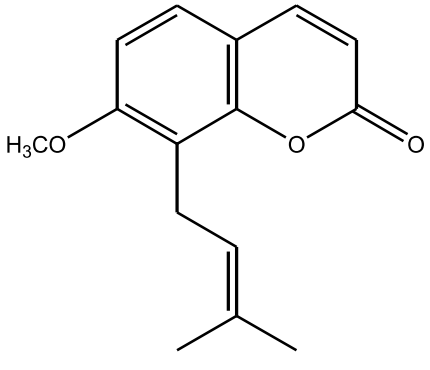

Figure 1. Structure of osthole.

1.0 g of osthole sample was weighed precisely. 10 ml of normal hexane, chloroform, acetyl acetate, ethanol, acetonitrile and water were added respectively. After 60 mins of sonification, the solution was filtered for later use. 5 mL of filtrate was added to 5 mL of internal standard. After well mixing, the mixture was filtered using 0.45 µm FP Vericel (PVDF) filter (Gelman Sciences). The filtrate was taken as sample liquid, which was further quantificated with gas chromatograph.

3. Preparation and identification of sample solution

1.0 g of osthole sample was weighed precisely and dissolved in the best solvent to make the final volume to 10 mL. After 60 mins of sonification, the solution was filtered for later use. 5 mL of filtrate was added to 5 mL of internal standard. After well-mixing, it was filtered using 0.45 µm FP Vericel (PVDF) filter (Gelman Sciences). The filtrate was taken as sample liquid, which was further analyzed with gas chromatograph.

4. Validation of quantification[15]

A. Linear concentration range identification

Osthole and dehydrated caffeine anhydrous were precisely weighed and prepared as osthole stock solution (2.0 mg/mL) and internal standard solution (1.0 mg/mL) after dissolving in proper amount of ethanol. Osthole stock solution was prepared in standard solutions of five different concentrations (800.0, 400.0, 200.0, 20.0 and 10.0 µg/mL) and each solution has the same concentration of internal standard of 500.0 µg/mL. The standard solutions were injected in GC and analyzed three times. Chromatograms were shown in Figure 2. Y-axis illustrates the ratio of the peak area of standard to internal standard, and X-axis shows the concentration of standard solutions. Linear calibration function and relative coefficients were obtained. The lowest measurable concentration equals 10 σ/S, σ is the standard deviation of the Y-intercept of the regression line and S is the slope of the calibration line.

B. Precision and accuracy

Osthole content of fresh standard solutions for preparation of calibration curve was measured at 8:00 am, 12:00 pm and 4:00 pm for three consecutive days. The osthole content's average, standard deviation, variation and relative difference were calculated to evaluate the stability of analytic condition, as well as the precision and recurrence of measured data.

C. Recovery assay

1.0 g of Cnidii Fructus with known osthole content was weighed precisely. Afterwards, 2, 4 and 6 ml of osthole stock solutions (1.0 mg/mL) and 10 mL of best solvent was

Table 1. Comparison of osthole purification efficiency between vacuum fractional distillation and column chromatography

Items	Vacuum fractional distillation	Column chromatography[6]
Apparatus	Vacuum distillation apparatus and vacuum pump	Glass column and fractional collector
Operation technique required	Non-skilled	Semi-skilled
Pollution	Negligible	A lot of waste solvent
Purity	100.02%	99.83%
Solvent required	Little	Much
Time	Short (< 8 hr)	Long (>> 8 hr)
Yield	85–92%	75–85%

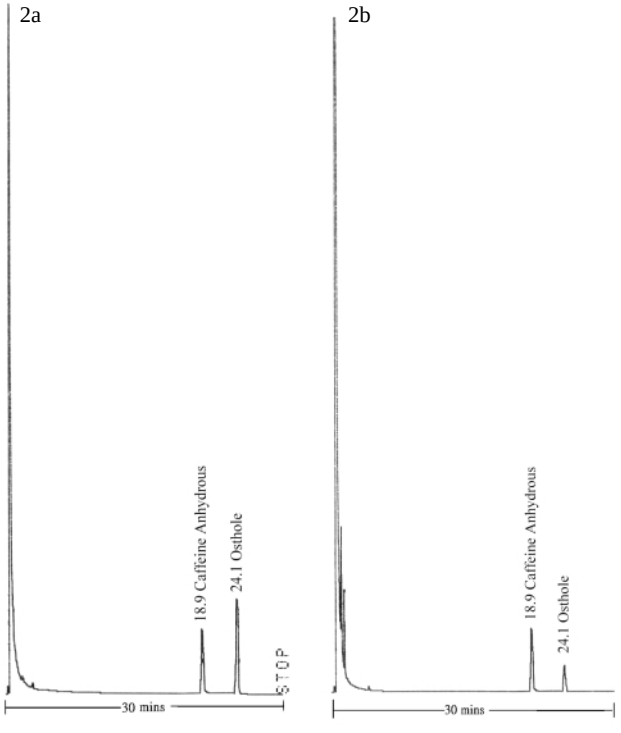

Figure 2. Chromatograms of osthole and its internal standard caffeine anhydrous in (2a) standard solution (osthole 0.4 mg/mL; caffeine anhydrous 0.5 mg/mL) and (2b) Cnidii Fructus.

added. The solutions were sonificated for 60 minutes and filtered for later use. 5mL of filtrate was added to and well mixed with 5 mL of internal standard. It was then filtered using 0.45 µm FP Vericel (PVDF) filter (Gelman Sciences). The filtrate was taken as sample solution, which was further analyzed with gas chromatograph.

Recovery(%)=[(measured osthole-known osthole)/total osthole added] ×100.

RESULTS AND DISCUSSIONS

1. Separation and purification of osthole

The boiling point of osthole in reduced pressure was not reported in literature. Separation and purification by vacuum fractional distillation is a better method than column chromatography when molecular structure was considered (Table 1). This fast and economical approach required only trace amount of diluted ethanol for recrystalization.

Not every organic compound can be separated by the vacuum fractional distillation. The molecular structure, molecular weight, resistance to thermo- decomposition and intra- or intermolecular hydrogen bond interaction should be taken into consideration[16]. This approach is carried on when saturated vapor from the distillated target is able to permeate the apparatus. There are risks with employing the vacuum fractional distillation, such as decomposition and carbonization. But this method will be a better choice than column chromatography when the target is clear and appropriate distillation parameters are adopted. It is also convenient to have a mass production. For the modernization of Chinese medicine, it is important to keep researching effective separation techniques for active ingredients.

Table 2. Effect of solvent types on extraction efficiency of osthole from Cnidii Fructus

Solvent	Extraction Efficiency (%)
n-Hexane	65.4
Chloroform	88.5
Ethyl acetate	83.4
Ethanol	100.0
Acetonitrile	92.2
Water	5.7

* Values in the right column are percentage relative to the best extraction efficiency value (100%).

2. Best solvent

Table 2 compares the efficiency of six solvents for extracting osthole. It shows that ethanol is the best.

3. Validation of quantification method

Calibration function for osthole, $Y=(4.249 \times 10^{-3})X-0.001$ ($r = 0.999$), which includes concentration ranging from 10.0 µg/mL to 800.0 µg/mL, showed excellent linear relationship. Precision and accuracy of chromatography are listed in Table 3. The result was satisfactory. The lowest measurable concentration was 10.0 µg/mL. The recovery efficiency ranges from 99.50% to 100.25% (Table 4).

4. Osthole content in commercial Cnidii Fructus products

After Cnidii Fructus was extracted in the best condition, osthole content of commercial Cnidii Fructus samples

Table 3. Intra-day and Inter-day analytical precision and accuracy of osthole determination

	Intraday (n=3)		Interday (n=3)	
Concentration (μg/mL)	Precision Mean ± S.D. (C.V.%)	Accuracy R.E. (%)	Precision Mean ± S.D. (C.V.%)	Accuracy R.E. (%)
800.0	797.7 ± 7.2 (0.9)	-0.3	819.8 ± 8.9 (1.1)	2.5
400.0	407.0 ± 2.5 (0.6)	1.8	402.4 ± 2.4 (0.6)	0.6
200.0	195.1 ± 1.5 (0.8)	-2.5	194.5 ± 1.2 (0.6)	-2.8
20.0	19.8 ± 0.2 (1.0)	-1.0	19.8 ± 0.1 (0.5)	-1.0
10.0	10.4 ± 0.2 (1.9)	4.0	10.2 ± 0.1 (1.0)	2.0

Table 4. Recovery of osthole from Cnidii Fructus

Osthole content (mg/g)	Amount added (mg/g)	Amount measured (Mean ± S.D., mg/g)	Recovery (%) (Mean ± S.D.)
21.9	2	1.99 ± 0.32	99.50 ± 1.6
21.9	4	4.01 ± 0.18	100.25 ± 4.5
21.9	6	6.12 ± 0.32	100.02 ± 5.3

Osthole content in Cnidii Fructus ranged from 0.3 to 24.4 mg/g (14).
S.D.:Standard deviation. n=3.

Table 5. Osthole content (mg/g) in various Cnidii Fructus samples of different sources

Sample No.	Source	Content (Mean ± S.D.)	R.S.D.(%)	Sample No.	Source	Content (Mean ± S.D.)	R.S.D.(%)
1	Vendor (1)	21.41 ± 0.30	1.4	6	Hu-Nan Province	18.42 ± 0.40	2.2
2	Vendor (2)	12.40 ± 0.13	1.0	7	Ann-Huei Province	14.60 ± 0.21	1.4
3	Vendor (3)	15.83 ± 0.70	4.4	8	Ho-Nan Province	11.21 ± 0.12	1.1
4	Vendor (4)	19.31 ± 0.32	1.7	9	Nei Mon-Ku (1)	4.51 ± 0.24	5.3
5	Vendor (5)	0.51 ± 0.02	3.9	10	Nei Mon-Ku (2)	0	

S.D.: Standard deviation, n=3, R.S.D. :Relative standard deviation.

from Taiwan was analyzed by GC with the conditions described above. The content varied from 0.05% to 2.14%. The osthole content of Cnidii Fructus from Mainland China increased southwardly. After referral to the constitution index[2] of Cnidii Fructus, Cnidii Fructus from Inner Mongolia should be *Cnidium dahuricum* (Jacq.) Turcz. ex Fisch et Mey. One purchased phony product was considered fruits of wild carrot and from which no osthole were detectable.

5. Comparison of quantification method

Pharmacopoeia of the People's Republic of China, 2000[8] suggested the specs of Cnidii Fructus. The osthole content was measured by thin layer chromatography and fluorescence quantification, which has worse accuracy than that of HPLC and GC[17]. Sagara et al[14] used HPLC to analyze the Cnidii Fructus composition in 1987. Since the composition of Cnidii Fructus is complicated, the column lifespan was shortened all the time[17]. In 1996, Tsai[18] studied the bioavailability of osthole by HPLC approach.

In 1990, Tsai et al[2] analyzed the ingredients of Cnidii Fructus with GC where the cetyl alcohol was chosen as an internal standard. Cetyl alcohol belongs to the aliphatic alcohol family. It is wildly used as typical GC standards but notoriously unstable[19]. This study employed the capillary gas chromatography in osthole content analysis. The samples required no purification pretreatment. The operation was convenient and precise. Caffeine anhydrous served as internal standard. It has high purity (>99.9%), easy access and resistance to thermo-decomposition.

CONCLUSION

1. It is efficient to separate and purify osthole from Cnidii Fructus extract with vacuum fractional distillation.
2. It is simple, precise and reliable to determine the osthole content by gas chromatography.
3. Commercial Cnidii Fructus samples varied in their osthole content a lot; the osthole content of Cnidii Fructus samples collected from Southern China was higher than those collected from Northern China.

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REFERENCES

1. Hsiu, K. G., Hou, H. S., Hsiu, L. S. and Gin, Z. R. 1996. Chinese Herbal. 1st ed. pp. 1124-1126. China Medical Technology Press. Nanjing, P.R.C.
2. Tsai, G. N., Hsiu, K. G., Gin, Z. Y., Hsiu, L. S. and Yu, P.Y. 1990. Advance Research in Cnidii Fructus. pp. 648-

672. China Medical College. Nanjing, P.R.C.
3. Shei, P. C. 1996. Report of Relative Affair about Chinese Medicine in Mainland China, Committee of Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, R.O.C.
4. Huang, R. L., Chen, C. C., Huang, Y. L., Hsieh, D. J., Hu, C. P., Chen, C. F. and Chang, C. 1996. Osthole increases glycosylation of hepatitis B surface antigen and suppresses the secretion of hepatitis B virus in vitro. Hepatol. 24: 508-515.
5. Guh, J. H., Yu, S. M., Ko, F. N., Wu, T. S. and Teng, C. M. 1996. Antiproliferative effect in rat vascular smooth muscle cells by osthole, isolated from *Angelica pubescens*. Eur. J. Pharmacol. 298: 191-197.
6. Teng, C. M., Lin, C. H., Ko, F. N., Wu, T. S. and Huang, T. F. 1994. The relaxant action of osthole isolated from *Angelica pubescens* in guinea-pig trachea. N-S. Arch. Pharmacol. 349: 202-208.
7. Ko, F. N., Wu, T. S., Liou, M. J., Huang T. F. and Teng, C. M. 1992. Vasorelaxation of rat thoracic aorta caused by osthole isolated from *Angelica pubescens*. Eur. J. Pharmacol. 219: 29-34.
8. The Pharmacopoeia Commission of P.R.C. 2000. Pharmacopoeia of the People's Republic of China. part I. pp. 256. Chemical Industrial Publisher. Bejing, P.R.C.
9. Camarda, L., Mazzola, P. and Sprio, V. 1986. Coumarins from the fruits of Cachrys Ferulacea. J. Nat. Prod. 50: 310.
10. Merck Research Laboratories, 1996. The Merck Index. 12th ed. pp. 1182-1183. Merck & CO., Inc. New Jersey, U.S.A.
11. Chinese Medicine Report Center of Bureau of National Medicine Ministry. 1986. Handbook of Indicated Component of Herbal Drug. pp. 792-793. People Health Publisher. Bejing, P.R.C.
12. Baba, K., Hamasaki, F., Tabata, Y. and Kozawa, M. 1985. Chemical studies on Chinese crude drug "She Chuang Zi". Shoyakugaku Zasshi 39: 282-290.
13. Chen, T.C. 1999. The Handbook of Chinese Medicine Chemical Working Standard. pp. 150-151. Chinese Medicine Technology Publisher. Bejing, P.R.C.
14. Sagara, K., Oshima, T., Sakamoto, S. and Yoshida, T. 1987. Determination of coumarins in *Cnidium monnieri* Fructus by high performance liquid chromatography. J. Chromatogr. 338: 448-454.
15. International Conference on Hormonization (ICH) Q2B. 1996. Guideline on the Validation of Analytical Procedures : Methodology.
16. Morrison, R.T. and Boyd, R.N. 1987. Alcohol in "Organic Chemistry". 5th ed. pp. 636-639, Allyn and Bacon, Inc. New York, U.S.A.
17. Snyder, L. R. and Kirkland, J. J. 1985. The Column in "Introduction to Modern Liquid Chromatography". 2nd ed. pp. 168-245. Euro-Asia Publisher. Taipei, R.O.C.
18. Tsai, T. H., Tsai, T. R., Chen, C. C. and Chen, C. F. 1996. Pharmacokinetics of osthole in rat plasma using high performance liquid chromatography. J. Pharmaceut. Biomed. 14: 749-753.
19. Nawar, W. W. 1985. Lipid in "Food Chemistry". pp. 139-244. Fennema, O. R. ed. Marcel Dekker Inc. New York, U.S.A.

蛇床子藥材中蛇床子素之分離暨氣相層析定量

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摘　要

本研究的目的是探討蛇床子藥材中蛇床子素（osthole）之分離與定量，建立氣相層析法檢測多種不同來源蛇床子藥材中蛇床子素之含量。分別以6種溶媒抽提蛇床子藥材，評估抽提效率，並比較真空減壓分段蒸餾法與傳統管柱法純化取得蛇床子素之效益。所建立之氣相層析法係以氮氣為移動相，流速為40 mL/min，分流比120：1，以DB^{TM}-5為分析管柱（30 m × 0.53 mm I.D.，1.5 μm），FID檢測器，升溫程式為起始溫度135˚C維持12 min，升溫段：12˚C /min，末段溫度215˚C維持20 min，蛇床子素之滯留時間為24.1 min，內部標準品為無水咖啡因（caffeine anhydrous），滯留時間為18.9 min。結果顯示以乙醇抽提蛇床子素之效率最佳，水的抽提效果最差。真空減壓分段蒸餾法較適合用於純化取得蛇床子素。台灣市售蛇床子之蛇床子素含量為2.14%至0.05%；而中國大陸南方蛇床子之蛇床子素含量較北方所產者為高。

關鍵詞：蛇床子，蛇床子素，氣相層析法