Determination of Synephrine and *N*-Methyltyramine in Zhishi and Zhike (Immature Citrus Fruits) by High-Performance Liquid Chromatography with Electrochemical Detection

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High-performance liquid chromatography (HPLC) with electrochemical detection was used to determine synephrine (SYN) and *N*-methyltyramine (NMT) in Zhishi (Kijitsu 枳実) and Zhike (Kikoku 枳殻). This electrochemical detection method has not only specific selectivity but also very high sensitivity, thus facilitating the determination of SYN and NMT from pulverized Zhishi and Zhike samples. Zhishi and Zhike derived from *Citrus aurantium* produced in different districts in China were analysed. This method was found useful for evaluating the quality of a crude drug such as *Citrus aurantium*.

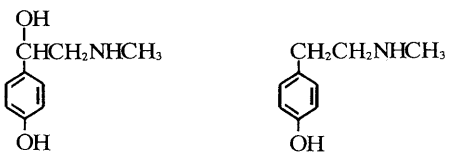
Keywords synephrine; *N*-methyltyramine; *Citrus aurantium*; Chinese crude drug; HPLC; electrochemical detection

Crude drugs derived from citrus fruits, whose Chinese names are Zhishi and Zhike (Japanese name: Kijitsu 枳実 and Kikoku 枳殻) are excellent Chinese medicinal materials considered to function as regulators of vital energy and to remove phlegm. In traditional Chinese medicine, they have been used as expectorants and antitussives. Zhishi is derived from unripe fruit and Zhike from the nearly-ripe fruit of *Citrus aurantium*. Both drugs contain synephrine (SYN) and *N*-methyltyramine (NMT) (Chart 1) which have numerous pharmacological activities such as raising blood pressure, increasing blood flow, and enhancing the contractive strength of heart muscle.[1-3] At present, the content of SYN is employed as a criterion for evaluating the quality of these crude drugs.

SYN in citrus crude drugs has been determined by different methods such as high-performance liquid chromatography (HPLC) with UV detection[4-6] and thin-layer chromatography (TLC)–densitometry detection.[7,8] NMT has been determined by HPLC with UV detection[5] and TLC.[8]

TLC requires development and coloration with a color reagent and this is time-consuming, even for semi-quantitative analysis. HPLC with UV detection requires the complex pretreatment of samples to avoid inteference by other substances.

Recently, HPLC with electrochemical detection has been used to determine SYN in insect brain.[9,10] This approach is characterized by specific selectivity with high sensitivity. NMT may be oxidized at a suitable potential, since it also possesses a phenolic hydroxyl group electrochemically oxidizable. HPLC with electrochemical detection was used in this study to simultaneously determine SYN and NMT in Zhishi and Zhike from different districts in China. This assay method should prove useful for measuring the content of NMT and SYN in various crude drugs derived from citrus fruits.



SYN NMT

Chart 1

Experimental

Plant Materials Experimental materials of *Citrus aurantium* were collected at several locations in China in June–July, 1989.

Chemicals and Reagents NMT hydrobromide was obtained from the Academy of Medicinal and Pharmaceutical Industries of Hunan in China. SYN was purchased from Sigma Chemical Co., and norepinephrine (NE) hydrochloride from Aldrich Chemical Co., powders of Chinese medicine extract from Tsumura Co., or Kanebo Co., and other chemicals from Wako Pure Chemical Industries, Ltd.

Chromatography The HPLC system consisted of a Jasco-880-PU pump (Jasco, Japan), 8125 injector fitted with a 50 μl injection loop (Rheodyne, U.S.A.), Kaseisorb LC-ODS-120-5 column (250 × 4.6 mm i.d., Tokyo Kasei, Japan), and EDP-1 type electrochemical detector (Kotaki, Japan). The mobile phase was prepared from 0.02 M citric acid–0.02 M NaH_2PO_4 (7 : 3, v/v) and adjusted to a final pH of 3.0 with citric acid. The system was operated at room temperature.

Sample Preparation Extraction Procedure A: Pulverized sample (1 mg) was extracted with 3 ml of methanol under ultrasonication for 30 min. After filtration, the residue was washed with 3 ml of methanol and the combined filtrate was evaporated to dryness. The residue was dissolved in 0.2 ml of 0.01 N HCl. To the solution, 0.5 ml of 2.0×10^{-10} M NE solution and 0.3 ml of the mobile phase were added to give a sample solution.

Extraction Procedure B: Pulverized sample (200 mg) was extracted with water (10 ml) at 90 °C for 6 h with occasional shaking. After filtration, the residue was re-extracted in the same mammer. The combined filtrate was applied onto a column of Amberlite IRC-50 (H^+ form) as reported by Kinoshita *et al.*[3] and Namba *et al.*[7] The column was washed with water and eluted with 5 N NH_4OH. The eluate was evaporated to dryness *in vacuo*. The residue was dissolved in 10 ml of 0.01 N HCl. The solution was diluted with the mobile phase to give a volume of 50 ml. A 0.25 ml of this solution was taken, then 0.5 ml of 2.0×10^{-10} M NE solution and 0.25 ml of mobile phase were added to give a sample solution.

Ten microliters of sample solution was injected into the HPLC column.

Results and Discussion

Hydrodynamic voltammograms are shown in Fig. 1. SYN and NMT each possess a hydroxyl group on the benzene nucleus, which is oxidized at a suitable potential. As seen in Fig. 1, NMT was oxidized at a potential above 0.8 V and SYN at above 0.9 V. Based on data from the hydrodynamic voltammograms, the detection potential for analysis of SYN and NMT was normally maintained at 1.2 V *vs.* Ag/AgCl. Ten microliters of standard solution mixture of NE as the internal standard compound, SYN and NMT were injected into the column followed by chromatography in the mobile phase pumped at a flow rate of 1 ml/min. The retention times of NE, SYN, and NMT were 3.4, 5.8 and 14.5 min, respectively (Fig. 2).

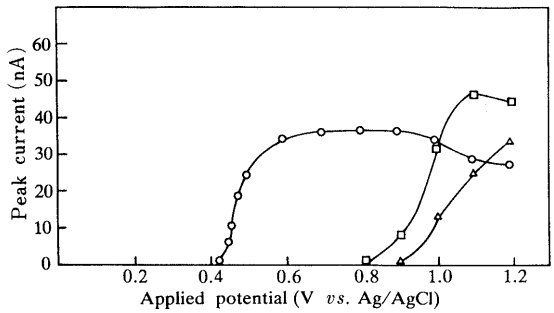


Fig. 1. Hydrodynamic Voltammograms of NE, SYN, and NMT

(○) NE, 80 pmol; (△) SYN, 72 pmol; (□) NMT, 170 pmol; chromatographic conditions: Column, Kaseisorb LC-ODS-120-5; mobile phase, a buffer (pH 3) of 0.02 M citric acid–0.02 M NaH_2PO_4 (7:3, v/v); sample volume, 10 μl; flow rate, 1.0 ml/min.

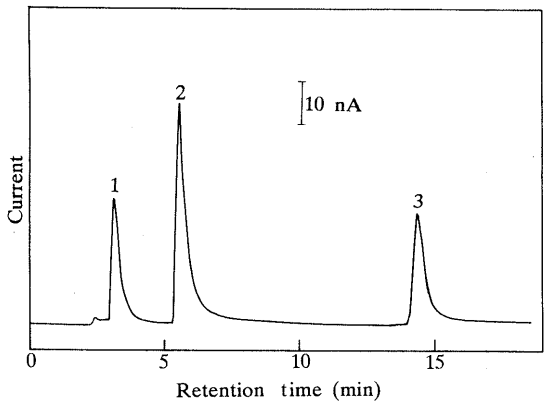


Fig. 2. Chromatogram of a Standard Mixture of SYN, NMT, and NE

Peaks: 1, NE (100 pmol, internal standard compound); 2, SYN (108 pmol); 3, NMT (80 pmol); applied potential: 1.2 V *vs.* Ag/AgCl; other chromatographic conditions are the same as those in Fig. 1.

TABLE I. Recovery of SYN and NMT Added to the Pulverized Xiang Zhike (Hunan)

Extraction procedure	Original content (ng/mg)		Content added (ng/mg)		Recovery of amount added (%)±S.D.[a]	
	SYN	NMT	SYN	NMT	SYN	NMT
A	1490	95	30	11	96±2	97±1
B	1420	95	33	12	97±1	98±1

a) ±Standard deviation ($n=4$).

A series of solutions containing the internal standard and various amounts of SYN and NMT was analysed. Peak height ratios relative to standard SYN and NMT were plotted against amounts of SYN and NMT. SYN in 17-10700 ng/ml was found to have a good linear relationship to peak height ratio with a correlation coefficient of 0.9981. In 270 ng/ml, relative standard deviation was 1.38% ($n=15$) and the detection limit ($S/N=3$) was 13 ng/ml. NMT in 12—26600 ng/ml indicated a good linear relationship to peak height ratio with a correlation coefficient of 0.9994. In 720 ng/ml, relative standard deviation was 1.44% ($n=15$) and the detection limit ($S/N=3$) was 12 ng/ml.

In the solvent for extracting the sample, 85% ethanol,[11] hot water (90—95 °C),[3,7] and methanol[12] have been used in some cases. SYN easily dissolves in methanol and thus

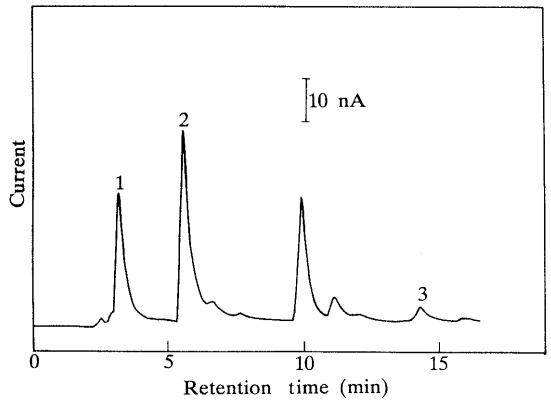


Fig. 3. Chromatogram of SYN and NMT Extracted from Xiang Zhishi (Hunan) According to Extraction Procedure A

Number of peaks and chromatographic conditions are the same as those in Fig. 2.

TABLE II. Contents of SYN and NMT in Zhishi and Zhike from Different Districts in China

Sample		Content			
		Extraction procedure			
Name	District (Province)	A		B	
		SYN (%)[b]	NMT (%)	SYN (%)	NMT (%)
Jiang Zhishi (江枳実)	Jiangxi (江西省)	0.245	0.014	0.242	0.011
Chuang Zhishi (川枳実)	Sichuan (四川省)	0.245	0.015	0.230	0.007
Xiang Zhishi (湘枳実)	Hunan (湖南省)	0.137	0.012	0.133	0.010
Chou Zhishi (臭枳実)	Jiangxi (江西省)	0.311	0.026	0.302	0.022
Xiang Zhishi (香枳実)	Jiangxi (江西省)	0.218	0.011	0.200	0.008
Jiang Zhike (江枳殻)	Jiangxi (江西省)	0.231	0.009	0.224	—[c]
Chuang Zhike (川枳殻)	Sichuan (四川省)	0.110	0.005	0.110	—[c]
Xiang Zhike[a] (湘枳殻)	Hunan (湖南省)	0.149	0.010	0.142	0.010
Chou Zhike[a] (臭枳殻)	Jiangxi (江西省)	0.124	0.010	0.124	—[c]
Xiang Zhike (香枳殻)	Jiangxi (江西省)	0.200	0.011	0.180	0.004

a) Cultural species. *b*) Relative standard deviation: Jiang Zhishi 1.5% ($n=7$), Xiang Zhishi (Hunan) 3.5% ($n=4$), Xiang Zhishi (Jiangxi) 4.8% ($n=4$), Jiang Zhike 5.0% ($n=7$), Chuang Zhike 4.5% ($n=4$), Xiang Zhike (Hunan) 5.2% ($n=4$), Xiang Zhike (Jiangxi) 3.8% ($n=4$). *c*) <0.001.

methanol extraction of the sample by ultrasonication was conducted in this study. For evaluation of practical application, methanol extraction (extraction procedure A) was compared with water extraction[3] (extraction procedure B). The recovery of SYN was essentially the same by both methods (Table I). Methanol was thus a more suitable solvent for sample extraction, and extraction procedure A, which is applicable to a small amount of sample, requires less time, and is convenient and very simple.

A typical chromatogram for the extracted sample solution is shown in Fig. 3. There was no interference with the peaks of SYN and NMT. SYN and NMT in Zhishi and Zhike from different districts in China were analysed and the

TABLE III. Content of SYN in Powders of Chinese Medicine Extract Produced in Japan

Name of powder	Content of SYN (%)	Remarks
Bukuryoin-gou-hangekouboku-to	0.067	5%[a]; 11%[b]
Chikujountan-to	0.047	7%[a]; 7%[b]
Chouto-san	0.060	11%[b]
Daisaiko-to	0.027	7%[a]
Junchou-to	0.022	8%[a]
Heii-san	0.078	22%[b]
Hochuekki-to	0.034	8%[b]
Keigairengyo-to	0.020	6%[a]
Kouso-san	0.037	19%[b]
Mashinin-gan	0.025	12%[a]
Seijouboufu-to	0.019	4%[a]
Sokeikakketsu-to	0.025	5%[b]
Tsudo-san	0.076	12%[a]; 8%[b]
Tokishigyaku-ka-goshuyu-shokyo-to	0.007	7%[c]

Content of *a*) Kijitsu (Immature Orange), *b*) Chinpi (Citrus Unshiu Peel), and *c*) Goshuyu (Evodia Fruit) in Chinese medicine used for the powders.

results are shown in Table II. Both were found to have anti-contraction activity and the effectiveness of the two drugs is indication of their amounts. Zhishi and Zhike usually contained SYN and NMT, and in most samples of Zhishi their amounts were higher than in Zhike. Their quantity in Chou Zhishi was higher than in other species.

The present method having high sensitivity was thus used to determine SYN in powders of Chinese medicine extracts available in Japan. The extraction of SYN was carried out by extraction procedure A and the results are shown in Table III. All samples contained extracts of Kijitsu, Chinpi (陳皮) and/or Goshuyu (呉茱萸), the content of each of which is shown in Table III. It has been reported that these three crude drugs usually contain synephrine of about 0.2%.[3,4,13]

In conclusion, HPLC with electrochemical detection makes possible the rapid, interference-free assay of SYN and NMT. It has high sensitivity and specific selectivity and can be conducted quite easily. It is suitable for determining SYN and NMT in immature citrus fruits as well as assessing the quality of Zhishi and Zhike.

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