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Full Length Research Paper

Discrimination of Zhishi from different species using rapid-resolution liquid chromatography-diode array detection/ultraviolet (RRLC-DAD/UV) coupled with multivariate statistical analysis

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Zhishi is one of the most commonly used traditional Chinese medicinal plants. Many species of *Citrus* plants, such as *Citrus aurantium* L. and its cultivars, *Citrus sinensis* Osbeck and its cultivars, and *Citrus junos* Sieb. ex Tanaka, can be used as Zhishi. However, Zhishi from different species exhibited differences in their therapeutic effects. In addition, it was very difficult to distinguish the species due to their similar morphologies. Flavanone compounds are the main bioactive constituents of Zhishi; in this study, flavanones were used as chemotaxonomic markers to distinguish among different *Citrus* samples. The contents of four flavanones (narirutin, naringin, hesperidin and neohesperidin) of 77 Zhishi samples from three *Citrus* species were determined using rapid resolution liquid chromatography-diode array detection/UV (RRLC-DAD/UV) and multivariate statistical analysis. Principal component analysis (PCA) of the quantitative data showed a clear separation of compositions among the three species of Zhishi samples. Furthermore, the established differential mode was subsequently applied to identify the origin of unknown commercial samples.

Key words: Zhishi, *Citrus* species, flavanone, rapid resolution liquid chromatography (RRLC), multivariate statistical analysis.

INTRODUCTION

Citrus plants are commonly used to derive medicine and/or food, and they contain a host of active flavonoids that contribute to health. For instance, the major species of *Citrus* that produce immature fruit used as Zhishi are *Citrus aurantium* L. and its cultivars, *Citrus sinensis* Osbeck and its cultivars, and *Citrus junos* Sieb. ex Tanaka (Xie, 1991; Cai et al., 1999). Zhishi (immature *Fructus aurantii*) is one of the earliest and most commonly used medicinal herbs in Traditional Chinese Medicine (TCM). The earliest record of Zhishi appeared in the Shen Nong Ben Cao Jing (A.D. 102-200). Zhishi is used to treat digestive disturbances (Chinese Pharmacopoeia, 2010). The herbs from different medicinal species and cultivation locations are widely used in clinical applications. As reported in the literature (Hao et al., 2002; Li et al., 1996), the effect of different species of Zhishi on enhancing intestinal transit speed are clearly different due to differences in the types and quantities of the chemical substances they contain. However, the morphologies of different species of commercial Zhishi are too similar to distinguish visually.

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Therefore, the development of a reliable analytical method for species differentiation among *Citrus* herbs is of great significance for ensuring the safety and effectiveness of clinical treatment. Flavonoids are very common and widespread secondary plant metabolites. They show a wide range of biological and physiological activities and usually serve as chemotaxonomic marker compounds (Wang et al., 2007; Zhou et al., 2006; Maria et al., 2007). Moreover, flavonoids, especially the flavanones contained in Zhishi, have been recognized as the main bioactive constituents. Flavanones in *Citrus* species, including narirutin, naringin, hesperidin and neohesperidin (Hu et al., 1994), are among the most prominent cancer-preventing agents (Albach et al., 1969; Castillo et al., 1992; Jourdan et al., 1985; Kawaii et al., 1999) and exhibit relatively high abundances. According to our previous research (Zhang et al., 2007) and the literature (Qin et al., 2009; Wu et al., 2009; Chuang et al., 2007), the flavonoid constituents of *Citrus* species vary according to the species and the cultivation locations of the *Citrus* plants. This variation in flavonoid constituents may influence the therapeutic effect of different medicines that are produced using different plants. For these reasons, the aims of this work were to develop a reliable analytical method using flavanones as marker compounds and to establish a multivariate statistical analysis technique that can be utilized to identify species of *Citrus* plants such as *C. aurantium* L. and its cultivars, *C. sinensis* Osbeck and its cultivars, and *C. junos* Sieb. ex Tanaka.

Many studies have focused on the differentiation of Zhishi by chemical compounds detection or fingerprints (Chuang et al., 2007; Kawaii et al., 1999; Maria et al., 2007; Nogata et al., 2006; Qin et al, 2009; Wang et al., 2007; Zhang et al., 2007). However, in fingerprint test, more compounds are needed to be verified, and in some cases, reference standards were unavailable. Also the determinations of fingerprints are time-consuming. In this study, four flavanone compounds (narirutin, naringin, hesperidin and neohesperidin) were used to be quantified in 77 samples of Zhishi, which were classified as three different *Citrus* species by RRLC-DAD/UV. Furthermore, this convenient and reliable analytical method coupled with multivariate statistical analysis was utilized to establish an effective differential mode for the different species of Zhishi samples. Finally, the application of this established analytical method to unknown samples enabled the classification of ten commercial Zhishi samples as three different *Citrus* species.

MATERIALS AND METHODS

Chemicals and materials

Methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). The other reagents were from Beijing Chemical Inc. (Beijing, China). The naringin and hesperidin standards were purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). The narirutin and neohesperidin standards were obtained from Sigma-Aldrich (Tokyo, Japan).

The 77 authentic Zhishi samples were collected from four local provinces of China: Jiangxi, Sichuan, Zhejiang and Guizhou. They all were identified as genuine samples of *C. aurantium* L. and its cultivars (*CA*), *C. sinensis* Osbeck and its cultivars (*CS*), and *C. junos* Sieb. ex Tanaka (*CJ*), respectively, by Professors Ge Fei, Yan Zhuyun, Zhang Yungui, Xu Jianguo and Ke Fuzhi. The dried voucher specimens (marked as *CA*-1~*CA*-32, *CS*-1~*CS*-33 and *CJ*-1~*CJ*-12) were deposited at the Institute of Basic Theory, China Academy of Chinese Medical Sciences, Beijing, P. R. China. The ten commercial Zhishi samples (marked as CM-1~CM-10) were purchased from drug stores in different provinces of China.

RRLC-DAD/UV analysis

The data were obtained using an Agilent 1200 Series RRLC with DAD. The analytical conditions for recording chromatograms of the marker compounds in Zhishi were as follows: A Zobax Extend-C_{18} column (4.6 × 50 mm; 1.8 µm; Agilent Technologies) was used. The mobile phase was a mixture of methanol-2% HAc (30: 70, v/v) at a flow rate of 1.0 mL/min, and the column temperature was set to 25°C. The detection wavelength was set to 285 nm and the run time of chromatography was 8 min.

Sample preparation

Each pulverized dried sample (0.1 g) was refluxed with methanol (25 mL) for 30 min at 80°C. Then the supernatant solution was filtered through a 0.2 µm Millipore filter for the RRLC analysis. Each sample was prepared in triplicate. The injection volume was 2 µL. The calibration curves, precisions, and recoveries for the analyses were examined and validated. The limits of detection (Signal/Noise = 3) under the present conditions were 0.35 ~ 0.48 ng.

Statistical analysis

The RRLC-DAD/UV data for different species of Zhishi samples were analyzed to identify potential discriminant variables. A quantitative data set, which consisted of values taken from the RRLC analyses of the 77 samples, was used for the multivariate analysis. Multivariate statistical analyses, including unsupervised principal component analysis (PCA) and supervised partial least squares (PLS), were performed using the SAS 9.1.3 statistical package (order no. 195557). PCA was used to observe the natural interrelationship among the chemical components for each of the three *Citrus* species, which could be applied to differentiate the three species according to the differentially expressed components. Furthermore, differential mode was carried out through the use of a more sophisticated PLS. The critical *p* value for all analyses in this study was set to 0.05.

RESULTS AND DISCUSSION

Characteristics of the collected samples

For all 77 Zhishi samples, the original plant, local name, location of collection, year and growing environments are listed in Table 1. All of the samples were collected from the main Zhishi-producing provinces of China during a three-year period. According to previous results (Zhou et

Table 1. The origins of the 77 Zhishi samples collected.

No.	Original plant	Local name	Location and time of collection	Growing environment
***Citrus aurantium* L.**				
CA-1	*C. aurantium* cv *Xiucheng*	Xiucheng	Xingan, Jiangxi; 2007	Plain (N 27° E 115°; Alt.20~30 m)
CA-2	*C. aurantium* cv Xiucheng	Xiucheng	Qingjiang, Jiangxi; 2007	Hillsides (N 27° E 114°; Alt.100~200 m)
CA-3~*CA*-10	*C. aurantium* cv Xiucheng	Xiucheng	Xingan, Jiangxi; 2008	Hillsides (N 27° E 115°; Alt.50~60 m)
CA-11~*CA*-21	*C. aurantium cv* Xiucheng	Xiucheng	Xingan, Jiangxi; 2009	Hillsides (N 27° E 115°; Alt.50~60 m)
CA-22	*C. aurantium* cv Jizicheng	Jizicheng	Zhangshu, Jiangxi; 2009	Plain (N 27° E 115°; Alt.20~30 m)
CA-23~*CA*-24	*C. aurantium* × *P. trifoliata*	Citrange	Yuanjiang, Hunan; 2007	Plain (N 28° E 112°; Alt.30~40 m)
CA-25~*CA*-27	*C. aurantium* L.	Sour orange	Jiangjin, Sichuan; 2008	Field margins (N 29° E 106°; Alt.200~230 m)
CA-28	*C. aurantium* L.	Sour orange	Jiangjin, Sichuan; 2007	Hillsides (N 29° E 106°; Alt.231 m)
CA-29	*C. aurantium* L.	Jiangjin sour orange	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.231 m)
CA-30~*CA*-31	*C. aurantium cv* Daidai	Daidai	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.229 m)
CA-32	*C. aurantium* cv Morocco sour orange	Morocco sour orange	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.45 m)
***Citurs junos* Sieb.ex Tanaka**				
CJ-1~*CJ*-4	*C. junos* Sieb. ex Tanaka	Xiangcheng	Xingan, Jiangxi; 2008	Hillsides (N 27° E 115°; Alt.50~60 m)
CJ-5~*CJ*-6	*C. junos* Sieb. ex Tanaka	Tuanye Xiangcheng	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.220 m)
CJ-7~*CJ*-12	*C. junos* Sieb. ex Tanaka	Xiangcheng	Xingan, Jiangxi; 2009	Hillsides (N 27° E 115°; Alt.50~60 m)
***Citrus sinensis* Osbeck**				
CS-1	*C. sinensis* Osbeck	Sweet orange	Jiangjin, Sichuan; 2007	Field margins (N 29° E 106°; Alt.200~230 m)
CS-2	*C. sinensis* Osbeck	Sweet orange	Fengjie, Sichuan; 2008	Field margins (N 29° E 106°; Alt.200~230 m)
CS-3~CS-4	*C. sinensis* Osbeck	Sweet orange	Jiangjin, Sichua; 2008	Field margins (N 29° E 106°; Alt.200~230 m)
CS-5	*C. sinensis* Osbeck cv Jin Cheng	Jin Cheng	Qinglong, Guizhou; 2008	Hillsides (N 25° E 105°; Alt.1200~1300 m)
CS-6	*C. sinensis* Osbeck cv Navel orange	Navel orange	Qinglong, Guizhou; 2008	Hillsides (N 25° E 105°; Alt.1200~1300 m)
CS-7	*C. sinensis* Osbeck cv Blood orange	Blood orange	Qinglong, Guizhou; 2008	Hillsides (N 25° E 105°; Alt.1200~1300 m)
CS-8	*C. sinensis* Osbeck cv Valencia	Valencia	Qinglong, Guizhou; 2008	Hillsides (N 25° E 105°; Alt.1200~1300 m)
CS-9	*C. sinensis* Osbeck cv Blood orange	Blood orange	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-10	*C. sinensis* Osbeck cv Valencia	Valencia	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-11	*C. sinensis* Osbeck cv Navel orange	Navel orange	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-12	*C. sinensis* Osbeck cv Peng an 100	Peng an 100	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-13	*C. sinensis* Osbeck cv Li Cheng	Li Cheng	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-14	*C. sinensis* Osbeck cv Hong 6-6	Hong 6-6	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-15	*C. sinensis* Osbeck cv Feng Chan Ji Cheng	Feng Chan Ji Cheng	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-16	*C. sinensis* Osbeck cv Da Zhou Zao Shu	Da Zhou Zao Shu	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.239 m)
CS-17	*C. sinensis* Osbeck cv Chang Ye Cheng	Chang Ye Cheng	Jiangjin, Sichuan; 2009	Hillsides (N 29° E 106°; Alt.238 m)
CS-18	*C. sinensis* Osbeck cv Yin Zao Cheng	Yin Zao Cheng	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)

Table 1. Contd.

CS-19	*C. sinensis* Osbeck cv Omishima navel orange	Omishima navel orange	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-20	*C. sinensis* Osbeck cv Delta Valencia	Delta Valencia	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-21	*C. sinensis* Osbeck cv Liu Ben Cheng	Liu Ben Cheng	Huangyan, Zhejiang; 2009	Plain (N 28° E 121°; Alt.7 m)
CS-22	*C. sinensis* Osbeck cv Early Gold sweet orange	Early Gold sweet orange	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-23	*C. sinensis* Osbeck cv Washington Sanguine	Washington Sanguine	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-24	*C. sinensis* Osbeck cv Olinda Valencia	Olinda Valencia	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-25	*C. sinensis* Osbeck cv Seike navel orange	Seike navel orange	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-26	*C. sinensis* Osbeck cv Feng Chan Ji Cheng	Feng Chan Ji Cheng	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-27	*C. sinensis* Osbeck cv Hamlin	Hamlin	Huangyan, Zhejiang; 2009	Plain (N 28° E 121°; Alt.7 m)
CS-28	*C. sinensis* Osbeck cv Jin Cheng	Jin Cheng	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-29	*C. sinensis* Osbeck cv Navel orange	Navel orange	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-30	*C. sinensis* Osbeck cv Midknight Valencia	Midknight Valencia	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-31	*C. sinensis* Osbeck cv Hong Jiang Cheng	Hong Jiang Cheng	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-32	*C. sinensis* Osbeck cv Ming Liu Cheng	Ming Liu Cheng	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)
CS-33	*C. sinensis* Osbeck cv Fukumoto navel orange	Fukumoto navel orange	Huangyan, Zhejiang; 2009	Hillsides (N 28° E 121°; Alt.40~60 m)

al., 2006; Maria et al., 2007), the concentrations of naringin and neohesperidin in the fruits of *C. aurantium* are at a maximum during the logarithmic phase of growth,gradually decreasing until the organs reach maximum development. Thus, in this study, the diameters of collected Zhishi samples were limited to 0.5 cm~2.5 cm, which is also consistent with the requirements of the Chinese Pharmacopoeia (Chinese Pharmacopoeia, 2010). Thirty-two samples (*CA*-1~*CA*-32) were identified as *C. aurantium* and its five cultivars (*C. aurantium* cv Xiucheng, *C. aurantium* cv Jizicheng, *C. aurantium* × *P. trifoliata, C. aurantium* cv Daidai, and *C. aurantium* cv Morocco sour orange); 12 samples (*CJ*-1~*CJ*-12) were identified as *C. junos*; and 33 samples (*CS*-1~*CS*-33) were identified as *C. sinensis* and its cultivars. The cultivars of *C. sinensis* are abundant in China, and 12 kinds of *C. sinensis* and its cultivars were collected in this study, as shown in Table 1.

Method validation

Calibration curves were prepared by plotting the peak area of marker compounds against the corresponding concentrations. Good linear relationships (R^2 = 0.9998 for naringin, and 0.9999 for narirutin, hesperidin and neohesperidin) are demonstrated over a range of 4~50 μg/mL. Limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined on the basis of response and slope of each regression equation at a signal to noise ratio of 3 and 10, respectively.

The LOD for the four marker compounds ranged from 0.04 to 0.25 μg/mL, and the corresponding LOQ ranged from 0.11 to 0.69 μg/mL. The accuracy of the analytical method was evaluated using the recovery test. The mean recoveries are from 99.9 to 102.1% with RSD less than 2.01% for the four reference compounds.

The precision of the assay was determined by repeatability. The RSD of retention time and peak area ranged from 0.34 to 1.91% and 1.14 to 1.96%, respectively.

RRLC-DAD/UV analyses of the three different species of Zhishi samples

In the 32 samples of *CA*, all of the four reference standards could be identified. The concentrations of narirutin, naringin, hesperidin and neohesperidin in this species of Zhishi samples are shown in Table 2.

The concentrations of narirutin and hesperidin ranged from 0.41 to 1.12% and 0.41 to 1.96%,

Table 2. The concentrations of the four flavanones investigated in the 77 Zhishi samples from three species of *Citrus* plants.

No.	Narirutin		Naringin		Hesperidin		Neohesperidin		Total (%)	Naringin/Narirutin
	Content	RSD (%)	Content	RSD (%)	Content	RSD (%)	Content	RSD (%)		
CA-1	0.92	1.3	10.94	1.2	0.79	0.25	10.16	0.45	22.81	11.84
CA-2	0.61	0.64	7.23	1.1	1.65	0.33	17.10	0.74	26.59	11.83
CA-3	0.59	1.1	7.01	2.4	1.26	0.57	14.80	2.0	23.66	11.84
CA-4	0.51	0.17	8.14	0.25	1.96	0.58	12.70	1.9	23.31	15.96
CA-5	0.58	0.28	6.21	0.55	1.32	0.48	17.00	0.91	25.11	10.67
CA-6	0.80	1.6	11.90	0.74	0.96	1.1	18.40	0.97	32.06	14.86
CA-7	0.88	2.1	11.20	0.56	1.18	2.5	17.50	0.47	30.76	12.68
CA-8	0.81	0.89	10.10	0.24	1.72	0.25	11.80	0.24	24.43	12.41
CA-9	0.61	0.74	7.94	2.1	0.75	1.5	15.30	0.57	24.60	12.97
CA-10	0.41	0.62	6.05	0.36	0.66	0.25	12.17	0.36	19.30	14.63
CA-11	0.90	1.3	13.57	0.69	0.96	0.48	12.18	0.17	27.61	15.04
CA-12	0.48	0.14	9.74	0.61	0.54	0.51	12.13	0.28	22.90	20.09
CA-13	0.83	0.27	9.76	0.25	1.36	0.33	20.21	1.7	32.15	11.82
CA-14	0.64	1.2	12.52	0.25	0.54	0.69	16.03	2.1	29.74	19.43
CA-15	0.64	2.9	10.35	1.2	0.41	0.94	8.38	1.0	19.78	16.27
CA-16	0.66	1.2	10.81	2.1	0.38	0.28	9.21	0.36	21.06	16.35
CA-17	0.57	1.0	10.10	2.2	0.48	0.47	6.54	0.60	17.69	17.64
CA-18	0.44	0.64	9.69	1.2	0.43	0.77	8.95	0.14	19.51	21.88
CA-19	0.62	1.1	10.06	1.0	0.72	0.25	12.52	0.51	23.92	16.24
CA-20	0.46	2.1	8.69	2.5	0.41	0.62	8.96	0.58	18.52	18.83
CA-21	0.55	0.33	11.08	0.25	0.59	0.24	10.32	0.34	22.54	20.07
CA-22	0.66	1.8	9.23	0.54	0.89	0.28	16.52	0.21	27.30	13.91
CA-23	0.59	0.21	8.24	1.2	0.98	1.5	20.20	0.11	30.01	13.94
CA-24	0.87	1.9	12.70	0.58	1.76	0.66	9.48	0.28	24.81	14.55
CA-25	0.64	0.36	9.47	1.2	1.18	0.48	24.30	0.10	35.59	14.77
CA-26	0.55	0.48	9.06	0.52	1.21	0.41	27.25	0.97	38.07	16.41
CA-27	0.53	1.7	8.57	0.28	1.42	0.36	25.15	0.24	35.67	16.14
CA-28	0.59	1.5	11.50	1.2	0.52	0.58	19.10	0.28	31.42	19.62
CA-29	0.76	1.9	11.40	0.25	1.50	0.47	24.41	0.46	38.07	15.07
CA-30	1.01	0.24	13.35	2.2	0.99	0.54	22.68	0.97	38.04	13.23
CA-31	0.91	0.18	12.14	0.25	1.39	1.5	27.30	0.77	41.74	13.31
CA-32	1.12	0.25	14.63	1.25	1.23	0.65	23.12	0.45	40.10	13.08
CJ-1	13.20	1.6	0.15	0.25	12.40	1.5	0.12	0.62	25.88	0.01
CJ-2	14.80	1.7	0.38	1.2	12.10	1.8	0.50	0.24	27.79	0.03
CJ-3	12.30	2.3	0.19	0.25	10.90	0.36	0.17	0.48	23.56	0.02

Table 2. Contd.

CJ-4	13.30	0.69	0.14	1.5	13.20	0.61	0.13	0.15	26.77	0.01
CJ-5	6.99	0.22	5.32	0.36	7.51	0.47	6.63	0.25	26.45	0.76
CJ-6	4.85	0.55	2.94	0.96	4.99	1.3	3.10	0.87	15.90	0.61
CJ-7	16.12	0.14	0.27	0.58	9.46	1.2	0.11	0.14	25.96	0.02
CJ-8	15.66	0.14	0.14	2.1	8.77	2.5	0.09	0.16	24.67	0.01
CJ-9	16.23	0.36	1.66	0.25	12.15	1.4	2.15	0.61	32.19	0.10
CJ-10	15.67	1.5	0.31	0.24	13.50	1.6	0.21	0.89	29.69	0.02
CJ-11	14.65	1.7	0.24	0.15	11.52	0.54	0.33	0.69	26.74	0.02
CJ-12	11.08	1.9	0.17	0.25	10.14	0.44	0.27	0.41	21.66	0.02
CS-1	0.74	0.66	ND	—	21.40	0.36	ND	—	22.14	—
CS-2	0.76	1.5	ND	—	21.90	0.48	ND	—	22.66	—
CS-3	1.58	0.24	ND	—	21.70	0.15	ND	—	23.28	—
CS-4	1.23	0.67	ND	—	24.30	0.67	ND	—	25.53	—
CS-5	0.92	0.36	ND	—	27.60	0.19	ND	—	28.52	—
CS-6	1.76	0.14	ND	—	19.70	0.97	ND	—	21.46	—
CS-7	0.56	0.25	ND	—	26.00	0.14	ND	—	26.56	—
CS-8	0.34	0.14	ND	—	27.30	1.6	ND	—	27.64	—
CS-9	1.45	0.69	ND	—	25.22	0.39	ND	—	26.67	—
CS-10	2.25	0.98	ND	—	19.55	0.75	ND	—	21.80	—
CS-11	3.50	0.87	ND	—	17.92	0.89	ND	—	21.42	—
CS-12	1.12	1.87	ND	—	18.70	0.14	ND	—	19.83	—
CS-13	1.32	2.0	ND	—	18.35	1.7	ND	—	19.66	—
CS-14	2.06	2.1	ND	—	17.93	1.3	ND	—	19.99	—
CS-15	2.99	1.7	ND	—	17.56	0.65	ND	—	20.55	—
CS-16	1.90	0.36	ND	—	16.54	0.61	ND	—	18.44	—
CS-17	1.20	0.54	ND	—	29.99	0.41	ND	—	31.19	—
CS-18	1.31	0.48	ND	—	17.44	0.39	ND	—	18.75	—
CS-19	0.71	1.1	ND	—	8.68	0.91	ND	—	9.38	—
CS-20	0.43	0.94	ND	—	23.89	0.14	ND	—	24.32	—
CS-21	0.40	0.22	ND	—	16.46	1.9	ND	—	16.86	—
CS-22	0.99	0.32	ND	—	19.47	2.4	ND	—	20.47	—
CS-23	0.16	0.14	ND	—	9.04	1.6	ND	—	9.21	—
CS-24	1.15	1.5	ND	—	25.32	0.24	ND	—	26.47	—
CS-25	2.28	2.1	ND	—	10.54	1.6	ND	—	12.82	—
CS-26	1.86	2.6	ND	—	10.48	0.27	ND	—	12.34	—
CS-27	1.12	1.6	ND	—	23.15	1.6	ND	—	24.28	—
CS-28	0.78	0.29	ND	—	18.72	2.4	ND	—	19.50	—

Table 2. Contd.

CS-29	1.61	0.25	ND	—	15.64	1.4	ND	—	17.25	—
CS-30	0.31	0.14	ND	—	13.84	2.0	ND	—	14.16	—
CS-31	0.45	0.36	ND	—	21.70	0.68	ND	—	22.15	—
CS-32	0.59	0.14	ND	—	25.62	1.1	ND	—	26.20	—
CS-33	2.05	0.58	ND	—	23.27	2.8	ND	—	25.32	—

ND = not detectable.

respectively; the levels of naringin and neohesperidin were found to be much higher than the other two standards, ranging from 6.05 to 14.62% and 6.54 to 27.3%, respectively. The total amount of the four compounds was 18.52 to 41.74%.

Compared to the *CA* samples, the 12 samples of *CJ* exhibited some salient differences. The narirutin and hesperidin concentrations were higher in the *CJ* samples, with concentrations ranging from 4.86 to 16.23% and 4.99 to 13.50%, respectively. However, the concentrations of naringin and neohesperidin were much lower in the *CJ* samples,

In the 33 samples of *CS*, only narirutin and hesperidin were detectable, while naringin and neohesperidin could barely be detected (Kawaii et al., 1999; Rouseff et al., 1987). The concentrations of narirutin and hesperidin ranged from 0.16 to 3.5% and 9.04 to 17.92%, respectively. The total amount of these two compounds was 9.21 to 31.19%.

These findings indicated that different kinds of flavanones occurred at various concentrations in the different *Citrus* species of Zhishi samples. Samples from the same species cultivated in different locations did not show differences with regard to the types and content of the four reference standards. Furthermore, a comparison of the relative concentrations of naringin to narirutin (naringin/narirutin) among the three *Citrus* species suggested that the ratio was much higher in the *CA* samples than in the *CJ* and *CS* samples, which was consistent with the reported literature (Hosoda et al., 1989; Nogata et al., 2006). Though there were some differences of the four compounds in different species of Zhishi, it is hard to make clear differentiation among different species of Zhishi. Therefore, the variation among the different *Citrus* species was further investigated in this study using the multivariate statistical analysis technique PCA.

Principal component analysis

To differentiate the *CA, CJ* and *CS* samples, an unsupervised pattern recognition method (PCA) was performed. A two-component PCA score plot of RRLC-DAD/UV data was utilized to depict the general variation of the marker flavanones among the three *Citrus* species of Zhishi samples. The clear separation of the three different species was observed in the PCA scores plot, where each coordinate represented a sample (Figure 1).

A two-component PCA model cumulatively accounted for 93% of variation. The PCA scores plot in Figure 1 could be divided readily into three distinct clusters (*CA, CJ* and *CS*), indicating that the concentrations and distribution of flavanones varied significantly in the different *Citrus* species. Each sample was represented as a point in a scores plot (*CA* = ★, *CJ* = ▲ and *CS* = ◆). The *CJ* samples and the others were clearly separated by the principal component 1 (PC1), whereas the *CA* and *CS* samples were clearly separated by the principal component 2 (PC2). This statistical method can be used as a powerful tool for the authentication of *Citrus* species of Zhishi samples. Furthermore, the differential mode produced by the SAS 9.1.3 software was established through the use of PLS to identify the species of unknown commercial Zhishi samples.

Identifying the species of unknown commercial Zhishi samples

Ten commercially available Zhishi samples (CM-1~CM-10) were purchased from drug stores in different provinces of China. It is very difficult to distinguish the *Citrus* species merely by examining their morphologies because they are extremely similar; therefore, the present study quantified all four flavanones (narirutin, naringin, hesperidin and neohesperidin) in the commercial samples using the method described previously, and the data are listed in Table 3. The quantitative data were analyzed using the established differential

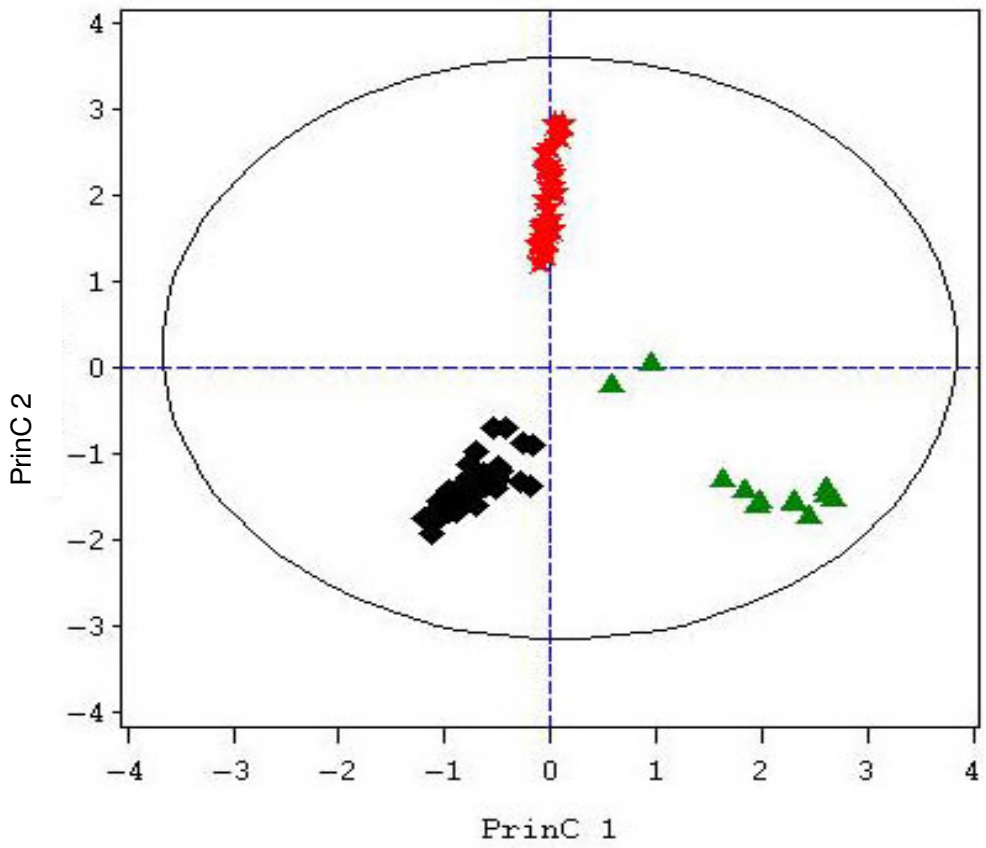

Figure 1. PCA scores plot (PC1 vs. PC2) of the three *Citrus* species of Zhishi samples: 32 samples from *C. aurantium* L. and its cultivars, 12 samples from *C. junos* Sieb. ex Tanaka and 33 samples from *C. sinensis* Osbeck and its cultivars.

Table 3. The concentrations of the four flavanones investigated in the ten commercial Zhishi samples.

No.	Location of purchase	Narirutin (%)	Naringin (%)	Hesperidin (%)	Neohesperidin (%)	Identified as species
CM-1	Guiyang, Guizhou province	2.42	ND	11.65	ND	*CS*
CM-2	Yantai, Shandong province	2.50	ND	10.25	ND	*CS*
CM-3	Dalian, Liaoning province	2.50	ND	13.62	ND	*CS*
CM-4	Beijing	1.49	ND	11.13	ND	*CS*
CM-5	Beijing	0.59	ND	6.30	ND	*CJ*
CM-6	Chongqing	0.97	ND	8.99	ND	*CS*
CM-7	Jiangjin, Sichuan province	1.27	12.05	1.31	12.68	*CA*
CM-8	Nanchang, Jiangxi province	0.61	7.23	1.65	17.12	*CA*
CM-9	Nanjing, Jiangsu province	0.69	11.54	0.92	16.05	*CA*
CM-10	Zhengzhou, Henan province	0.37	ND	11.36	ND	*CS*

ND = not detectable.

mode, and CM-1~CM-4, CM-6 and CM-10 were identified as *C. sinensis* and its cultivars. CM-5 was identified as *C. junos*, and CM-7~CM-9 was identified as *C. aurantium* and its cultivars, as shown in Figure 2.

In this study, a convenient and reliable analytical method coupled with multivariate statistical analysis was used to evaluate and distinguish the following three species of Zhishi samples: *C. aurantium* and its cultivars, *C. sinensis* and its cultivars, and *C. junos*. Four active flavanones (narirutin, naringin, hesperidin and neohesperidin)

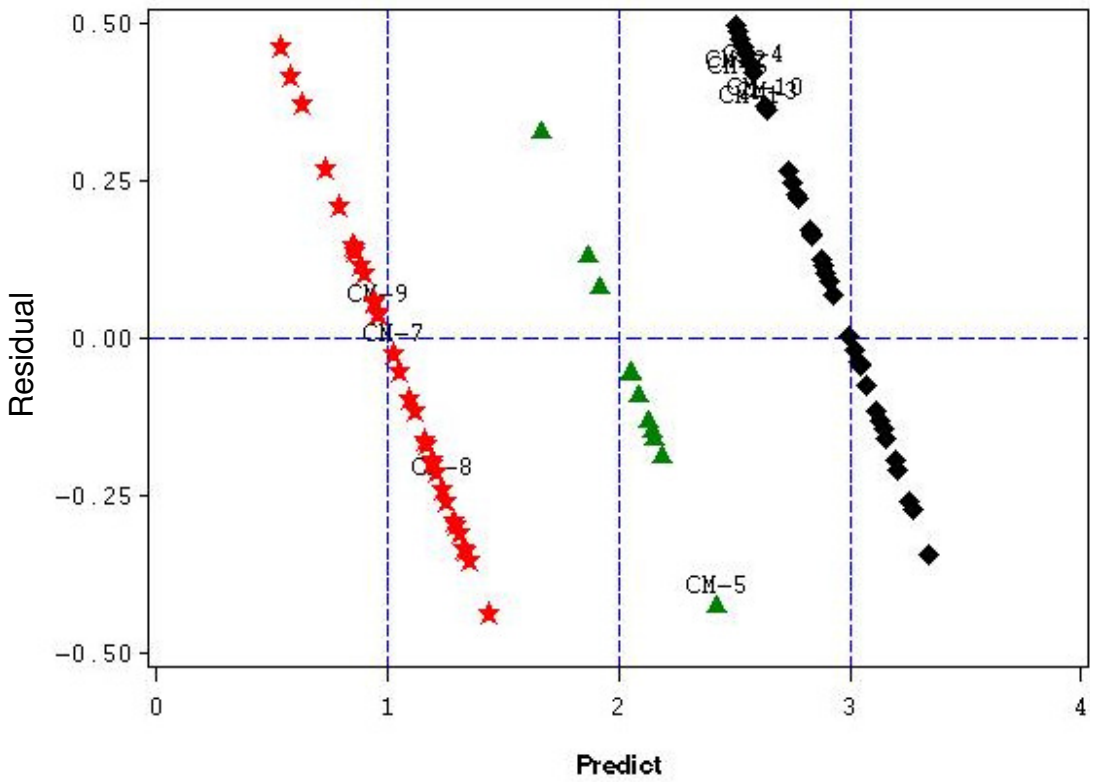

Figure 2. Identification of the ten unknown commercial samples through the use of PLS. (*CA* = ★, *CJ* = ▲ and *CS* = ◆).

were used as marker compounds, which were quantified using RRLC-DAD/UV, and the corresponding data were analyzed using PCA analysis. Using the methods described in the forgoing, 77 Zhishi samples were clearly identified as three *Citrus* species.

Though we did not know whether we can use less marker compounds for the differentiation of different species of Zhishi, the established differential mode by using the four important marker compounds could be applied to identify unknown commercial samples. Hence, this work is of great importance for the evaluation and authentication of Zhishi samples, which is ultimately of great significance for ensuring the safety and effectiveness of its clinical use.

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