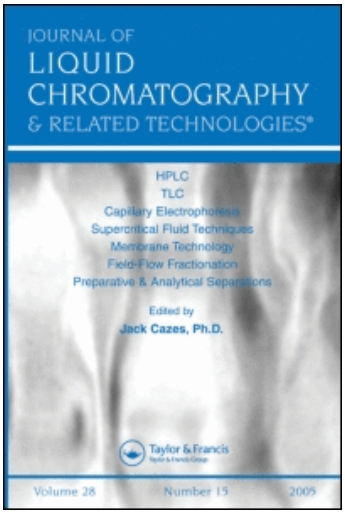

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Preparation of National Certified Reference Materials of Active Compounds from Natural Products by CCC

Tianyou Zhang[a]; Xueli Cao[a]; Xiao Han[a]
[a] Beijing Institute of New Technology Application, Beijing, China

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Preparation of National Certified Reference Materials of Active Compounds from Natural Products by CCC

Tianyou Zhang,* Xueli Cao, and Xiao Han

Beijing Institute of New Technology Application,
Beijing, China

ABSTRACT

The preparation of 12 National Certified Reference Materials of active compounds from natural products with countercurrent chromatography (CCC) are reported in this paper. They are kaempferol (**1**), isorhamnetin (**2**), quercetin (**3**), icariin (**4**), epigallocatechin-3-*o*-gallate (EGCG) (**5**), epicatechin-3-*o*-gallate (ECG) (**6**), gallocatechin-3-*o*-gallate (GCG) (**7**), resveratrol (**8**), piceid (**9**), 2,3,5,4′-tetra hydroxystibene-2-*o*-*D*-glucoside (**10**), puerarin (**11**), 3′-methoxy-puerarin (**12**). All of their certified purities were higher than 98%.

Key Words: Certified reference materials; Natural products; CCC; Homogeneity assessment; Stability assessment.

*Correspondence: Tianyou Zhang, Beijing Institute of New Technology Application, Beijing 100035, China; E-mail: zhangty@public.east.cn.net.

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INTRODUCTION

Natural products play an increasingly important role in pharmaceutical, cosmetic, flavor, and dietary supplement industries nowadays. The separation of active compounds from natural sources presents a number of difficulties. Often, the compounds of interest are present as minor components of extremely complex mixtures. Since adsorption chromatography plays a major role in obtaining a pure compound from complex mixtures, any new techniques that are rapid, and do not lead to denaturation, material loss, or artifact formation are highly welcome. Countercurrent chromatography (CCC), which is a liquid–liquid separation method and uses no solid support in column, has proven to be a very effective alternative to other preparative and semipreparative chromatographic methods during the past decade.

In China, we started our research on CCC from 1970s. We have developed a series of high-speed CCC (HSCCC) instruments and separation methods, such as semipreparative HSCCC, analytical HSCCC, multidimensional HSCCC, pH-zone refining CCC, and cross-axis CCC. In the field of applications, HSCCC has been successfully applied to the separation and purification of a wide variety of natural active compounds, including flavones, isoflavones, saponins, terpenoids, flavanols, alkaloids, flavanols, tannins, anthraquinones, tanshinone, resveratrol and piceid, carotenoids, and glycoproteins from natural plants and Chinese traditional herbs.[1]

With the increasing concern for human health and environmental protection, many natural products based on the bioactive constituents have been rapidly developed for medicinal use, or as functional foods, additives to cosmetic, and other daily products for personal and home care. This makes it imperative to establish and implement a quality control system at all stages of the manufacture to the point of sale, to assure the quality of these products. Standard reference materials play an important role in quality control. Compared to the source of natural products, standard reference materials of individual active compounds with high purity are in extremely short supply worldwide. Only a few of them are commercially available and at high prices. Standard materials are also of great importance in quality control of traditional Chinese medicines, as well as, in their modernization and regeneration.

The intense competition in the current scientific and technical and socioeconomic development is mainly represented by talented competition in patents and in standards. This has drawn much attention from the Chinese government.

Under the permission and support of the State Bureau of Quality and Technical Inspection and based on our previous work on CCC research and application, a series of high-purity substances of active compounds from medicinal plants and agroproducts have been developed by taking advantage

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of the capacity of CCC in the production of high purity substance in large quantities, high recovery, and good reproducibility. This paper presents the preparation and certification of 12 National Certified Reference Materials (CRMs) of active compounds from natural products. Table 1 lists all of the 12 compounds, their structure, and source. This is the first batch of national CRMs of natural active compounds in China.

EXPERIMENTAL

Instruments

Two models of coil planet centrifuges (CPC) have been used in the preparation of high pure individual compounds.

1. Model GS10A2; a multilayer coil was prepared by winding 1.6 mm I.D. tubing coaxially onto a spool-shaped column holder. The β value ranges from 0.5 to 0.75, and the total capacity is 210–230 mL.
2. Another model of CPC is equipped with two opposite coils. The coils were prepared by winding 1.6 mm I.D. tubing coaxially onto two spool-shaped column holder. The β value ranges from 0.5 to 0.75, and the total capacity is 485 mL.

HPLC analysis was performed on Shimadzu LC-10A HPLC system, a Shimadzu SPD-M10A photodiode array detector, and a Shimadzu SIL-10AD autosampler. The freeze dryer is from Christ, Germany.

Materials

Most of the starting materials were the commercially available crude extracts of different natural products enriched with target compounds. Some of them were extracted from original plants in our lab.

Preparation with High-Speed Countercurrent Chromatography

According to the polarity of the compounds, different solvent systems from less polar to polar were used in HSCCC separation of individual components, including systems composed of chloroform–methanol–water, hexane–ethyl acetate–water, ethyl acetate–ethanol (or methanol)–water, and ethyl acetate–butanol–water, as presented in Table 2. One hundred-300 mg crude sample was loaded in each separation. Figure 1 illustrates HSCCC separation chromatograms of seven compounds of interest. Separation of two

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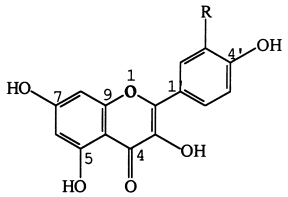
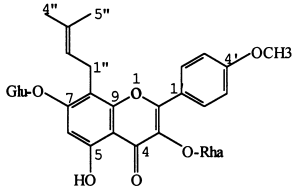
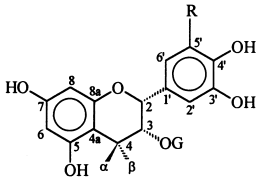
Table 1. Compounds and their sources.

	Compounds	Molecular structures	Sources
Flavones	Kaempferol (**1**) R = H Isorhamnetin (**2**) R = OCH Quercetin (**3**) R = OH		Leaves of *Ginkgo biloba* L.
	Icariin (**4**)		*Epimedium brevicornum* Maxim
Flavanols	Epigallocatechin-3-*o*-gallate (EGCG) (**5**) R = OH Epicatechin-3-*o*-gallate(ECG) (**6**) R = H		Green tea

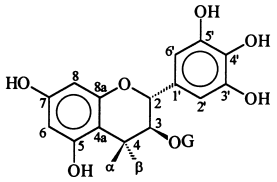
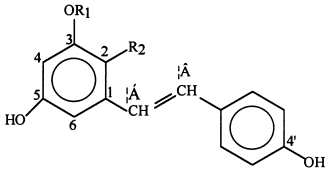
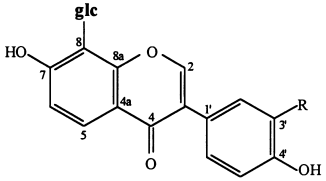
	Gallocatechin-3-*o*-gallate (GCG) (**7**)		
Stilbene compounds	Resveratrol (**8**) $R_1 = R_2 = H$ Piceid (**9**) R_1 = D-Glucose $R_2 = H$ 2,3,5,4′-Tetra hydroxystibene-2-*o*-D-glucoside (**10**) $R_1 = H$ (THSG) $R_2 = o$-*D*-Glucose		*Polygonum cuspidatum* Sieb.Et Zucc and grape seed. Polygonum multiflorum Thunb
Isoflavones	Puerarin (**11**) R = H 3′-Methoxy-puerarin (**12**) $R = OCH_3$		*Pueraria lobata* (willd.)Ohwi

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Table 2. Conditions of CCC separation and HPLC analysis.

Compounds	Solvent systems for HSCCC separation	HPLC analytical conditions
(**1**), (**2**)	Chloroform–methanol–water (4 : 3 : 2, v/v/v),	Column: Shim-pack CLC-ODS 150 × 6.0 mm; Mobile phase: Methanol/water (0.5% phosphoric acid) (50 : 50, v/v); Flow-rate: 1.5 mL/min; Column temperature: 30°C; Detection: UV368 nm.
(**3**)	Chloroform–methanol–water (4 : 3 : 2, v/v/v),	Column: Shim-pack VP-ODS 150 × 4.6 mm; Flow-rate: 1.0 mL/min. The others are the same as (**1**) and (**2**).
(**4**)	Chloroform–methanol–water (4 : 3 : 2, v/v/v),	Column: Phenomenex Luna 5 μ C18, 150 × 4.6 mm; Mobile phase: Methanol/water (60 : 40, v/v); Flow-rate: 1.0 mL/min; Column temperature: 35°C; Detection: UV270 nm.
(**5**), (**6**), (**7**)	Ethyl acetate–ethanol–water from 25 : 1 : 25 to 5 : 1 : 5, v/v/v, and hexane–ethyl acetate–water (1 : 4 : 5, v/v/v)	Column: Phenomenex Luna 5 μ C18, 150 × 4.6 mm; Mobile phase: A/B(1 : 1,v/v), A: acetic acid : methanol : water (1 : 1 : 98, v/v/v), B: Acetic acid : methanol : water: DMF (*N*,*N*-dimethyl flomamide) (1 : 1 : 48 : 50, v/v/v/v); Flow-rate: 1.0 mL/min; Column temperature: 30°C; Detection: UV280 nm.
(**8**)	Chloroform–methanol–water (4 : 3 : 2, v/v/v),	Column: Phenomenex Luna 5 μ C18, 150 × 4.6 mm; Mobile phase: Acetonitrile : water (30 : 70,v/v); Column temperature: 35°C; Flow-rate: 1 mL/min; Detection: 306 nm
(**9**)	Ethyl acetate–ethanol–water (100 : 1 : 100, v/v/v)	Mobile phase: 35% methanol in water gradient to 55% in 30 min. The others are the same as (**8**).

(continued)

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Table 2. Continued.

Compounds	Solvent systems for HSCCC separation	HPLC analytical conditions
(**10**)	Ethyl acetate–methanol–water (50 : 1 : 50, v/v/v)	Column: Shim-pack VP-ODS 250 × 4.6 mm; Column temperature: 30°C; Mobile phase: Methanol–water (2% acetic acid) (35 : 65,v/v); Detection: 254 nm.
(**11**), (**12**)	Ethyl acetate–butanol–water (2 : 1 : 3, v/v/v)	Column: Shim-pack VP-ODS 250 × 4.6 mm; Mobile phase: Methanol/water (2% acetic acid) (25 : 75,v/v); Flow-rate: 1 mL/min; Detection: 254 nm.

flavones (**1**) and (**2**), and three flavanols (**5**), (**6**), and (**7**) have been reported previously.[2,3]

After concentration of the target fraction under reduced pressure at room temperature and freeze drying, the dry pure sample was subjected to HPLC analysis, identification, and packaging.

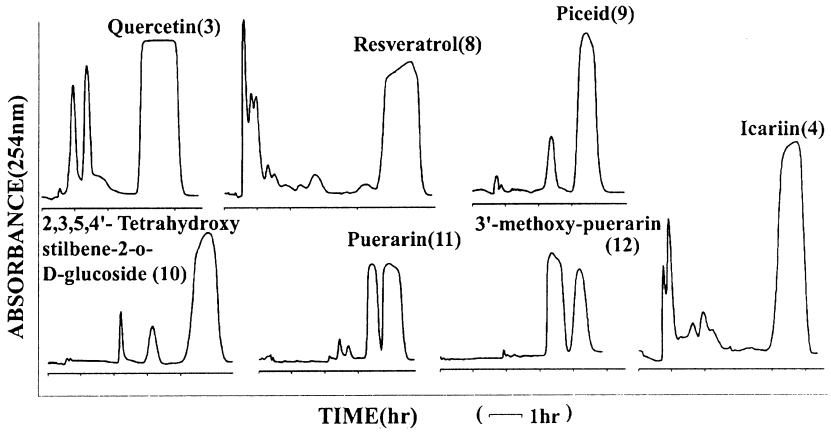

Figure 1. Partial CCC separation profiles of natural products. The liquid system can be chloroform–methanol–water (quercetrin, icariin, and resveratrol) or ethyl acetate–ethanol–water (piceid) or ethyl acetate–butanol–water (puerarin) and ethyl acetate–methanol–water (stilbene–glycoside). See Table 2 and original articles for full technical information.

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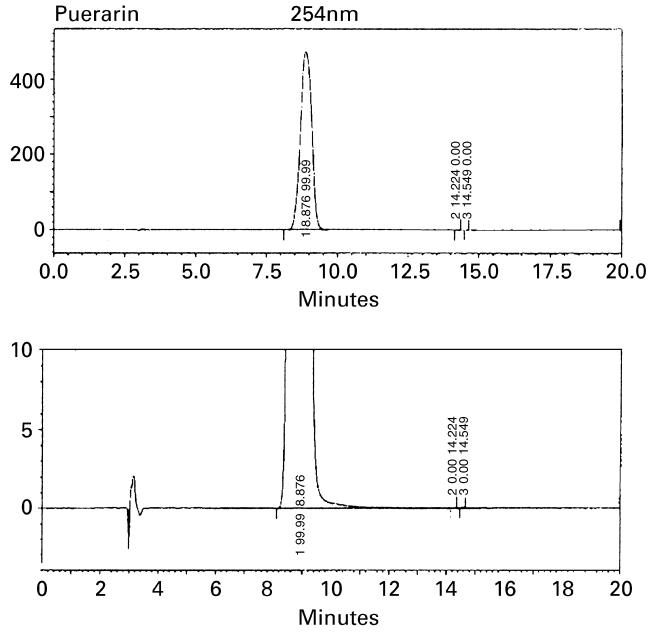

1:254nm, 8nm	Pk#	Retention Time	Area	Area Percent
1		8.876	13062306	99.99
2		14.224	606	0.00
3		14.549	614	0.00
Totals			13063527	100.00

Figure 2. HPLC chromatogram of puerarin. Column Shim Pack VD ODS C18 250 × 4.6 mm, 5 μm, mobile phase: methanol–water 25–75% v/v, flow rate 1 mL/min, detection UV at 254 nm.

HPLC Analysis of Purity

Before packaging, all the samples prepared from HSCCC were analyzed for purity by HPLC. The conditions of all HPLC analyses are listed in Table 2. Figure 2 gives the chromatogram of puerarin as an example of HPLC analysis. All samples were prepared in the concentration range 0.5–1.0 mg/mL, in which all possible trace residuals can be detected. The same HPLC analytical conditions were later applied in the test of homogeneity and stability, and final certification of standard samples.

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Identification by UV, IR, MS, NMR

Before packaging, all the samples prepared from HSCCC were also subjected to UV, IR, MS, ^{1}HNMR, and ^{13}CNMR characterization to assure that the samples were the target compounds.

Packaging and Storage

After HPLC analysis and structure elucidation, each item of CRM was collected in 200 mg/batch, and bottled in 5-mg units in a separate clean room. Forty units were obtained from each batch. Then the CRMs were sealed and stored below 4°C.

RESULTS AND DISCUSSIONS

Homogeneity Assessment

Homogeneity is an important property of CRMs. It is, nevertheless, a relative concept, closely related to the distribution of components in the material, the sample size, and the number of samples that have been selected to measure the homogeneity.

The homogeneity of the CRMs were assessed by comparing the HPLC analytical results, of purity of three samples, from each of 5–8 randomly selected bottles by the F test. The sample size was 1 mg. The results are summarized in Table 3. All the statistical F values were smaller than the critical $F_{0.05}$ values. This means, there is no statically significant difference between bottles and within a bottle, regarding the sample purity.

Certification of Purity of Each Certified Reference Material

The certification was accomplished by comparing the purity values of each CRM from two independent qualified laboratories. The certified values and their uncertainties were calculated statistically as below:

Data from Lab A: Xai, $i = 1, 2, 3, \ldots, n$, the mean value and its standard deviation were:

$$\overline{Xa} = \sum_{i=1}^{n} \frac{Xai}{n}, \quad S_a = \sqrt{\frac{\sum_{i=1}^{n} (Xai - \overline{Xa})^2}{n-1}}$$

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Table 3. Homogeneity assessment of each CRM.

Compounds	Purity, % Mean value	F value	$F_{0.05}(\gamma_1, \gamma_2)$
Kaempferol(**1**)	99.98 ($m=8$)[a]	2.01	$F_{0.05}(7,16)=2.66$
Isorhamnetin(**2**)	99.92 ($m=8$)	0.74	$F_{0.05}(7,16)=2.66$
Quercetin (**3**)	99.76 ($m=5$)	1.50	$F_{0.05}(4,10)=3.48$
Icariin (**4**)	98.75 ($m=8$)	2.61	$F_{0.05}(7,16)=2.66$
EGCG (**5**)	97.98 ($m=7$)	2.21	$F_{0.05}(6,14)=2.85$
ECG (**6**)	99.24 ($m=7$)	0.72	$F_{0.05}(6,14)=2.85$
GCG (**7**)	99.45 ($m=7$)	1.88	$F_{0.05}(6,14)=2.85$
Resveratrol(**8**)	99.93 ($m=5$)	0.77	$F_{0.05}(4,10)=3.48$
Piceid (**9**)	98.76 ($m=7$)	2.72	$F_{0.05}(6,14)=2.85$
THSG (**10**)	99.16 ($m=8$)	1.53	$F_{0.05}(7,16)=2.66$
Puerarin (**11**)	99.99 ($m=8$)	0.93	$F_{0.05}(7,16)=2.66$
3′-Methoxy-puerarin (**12**)	99.76 ($m=8$)	1.87	$F_{0.05}(7,16)=2.66$

[a]$m=$ number of selected sample bottles, three samples measured; $\gamma_1=m-1$, $\gamma_2=N-m$, $N=3m$.

Data from Lab B: Xbi, $i=1, 2, 3, \ldots, n$, the mean value, and its standard deviation were:

$$\overline{Xb}=\sum_{i=1}^{n}\frac{Xbi}{n},\quad S_b=\sqrt{\frac{\sum_{i=1}^{n}(Xbi-\overline{Xb})^2}{n-1}}$$

The mean value of the data from two labs and its standard deviation were calculated as

$$\bar{\bar{X}}=\frac{\bar{Xa}+\bar{Xb}}{2},\quad S_x{}^{-}=\sqrt{(\bar{Xa}-\bar{\bar{X}})^2+(\bar{Xb}-\bar{\bar{X}})^2}$$

The repetitive deviation of the data from two labs were calculated as:

$$Sr=\sqrt{\frac{Sa^2+Sb^2}{2}}$$

Finally, the certified value was reported as: $u=\bar{\bar{X}}$.

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Table 4. Certified purity of each CRM.

	Purity, %				
	Lab 1		Lab 2		
Compounds	X[a]	SD	X	SD	Certified values
Kaempferol (**1**)	99.98 ($n=10$)	0.01	99.77 ($n=10$)	0.01	99.9 ± 0.2
Isorhamnetin (**2**)	99.92 ($n=10$)	0.01	99.77 ($n=10$)	0.01	99.8 ± 0.1
Quercetin (**3**)	99.76 ($n=5$)	0.01	99.78 ($n=5$)	0.01	99.77 ± 0.02
Icariin (**4**)	98.68 ($n=7$)	0.09	98.73 ($n=7$)	0.10	98.71 ± 0.15
EGCG (**5**)	98.03 ($n=7$)	0.09	98.07 ($n=7$)	0.06	98.05 ± 0.12
ECG (**6**)	99.23 ($n=7$)	0.05	99.15 ($n=7$)	0.03	99.19 ± 0.12
GCG (**7**)	99.52 ($n=7$)	0.06	99.43 ($n=7$)	0.04	99.48 ± 0.14
Resveratrol (**8**)	99.93 ($n=5$)	0.02	99.93 ($n=5$)	0.02	99.93 ± 0.02
Piceid (**9**)	98.63 ($n=7$)	0.10	98.64 ($n=7$)	0.21	98.64 ± 0.24
THSG (**10**)	99.17 ($n=7$)	0.15	99.18 ($n=7$)	0.15	99.18 ± 0.22
Puerarin (**11**)	99.99 ($n=7$)	0.01	99.99 ($n=7$)	0.01	99.99 ± 0.02
3′-Methoxy-puerarin (**12**)	99.76 ($n=7$)	0.01	99.76 ($n=7$)	0.02	99.76 ± 0.03

Note: SD, standard deviation.
[a]X-mean value of selected sample, each sample is triplicate measured.

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The uncertainty associated with the certified value was reported as 1.96*S*, where:

$$S = \sqrt{S_x{}^{-2} + Sr^2 \frac{n-1}{n}} = \sqrt{S_x{}^{-2} + \frac{1}{2} Sr^2}$$

which is at the 95% level of confidence.

The certified value of each CRM purity has been summarized in Table 4. The results indicated that all the CRM purity could reach 98% or over.

The uncertainties associated with the certified purities of kaempferol (**1**) and isorhamnetin (**2**) were reported as $S_x{}^{-}$.

Assessment of Stability

All the CRMs were kept in 1 mL brown glass bottles and stored below 4°C. The stability during long-term storage was assessed by the same HPLC method as above. The results were presented in Table 5. For kaempferol (**1**) and isorhamnetin (**2**), no significant difference was observed between the purity data during one-year storage. For the other ten CRMs, as the certification was just finished not long ago, only two or six months stability was available. Stability during longer-term storage is still under investigation.

Table 5. Stability assessment of each CRM.

	Purity, %				
Compounds	1 week	1 month	2 months	6 months	1 year
Kaempferol (**1**)	99.96	99.97	99.97	99.99	99.98
Isorhamnetin (**2**)	99.91	99.91	99.90	99.92	99.92
Quercetin (**3**)	99.78	99.76	99.76	99.76	n.a.
Icariin (**4**)	98.97	98.96	98.76	98.95.	n.a.
EGCG (**5**)	98.07	98.00	97.99	n.a.	n.a.
ECG (**6**)	99.20	99.12	99.13	n.a.	n.a.
GCG (**7**)	99.42	99.42	99.37	n.a.	n.a.
Resveratrol (**8**)	99.93	99.93	99.94	99.92	n.a.
Piceid (**9**)	98.76	98.80	98.72	98.75.	n.a.
THSG (**10**)	99.18	99.16	99.16	99.15.	n.a.
Puerarin (**11**)	99.99	99.99	99.99	99.98	n.a.
3′-Methoxy-puerarin (**12**)	99.78	99.77	99.76	99.76	n.a.

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CONCLUSIONS

The above reported the preparation of the first batch of national CRMs of natural active compounds in China with HSCCC, and the certification of their purity. All the CRM purities could reach 98% or over just after a one step HSCCC separation. High-Speed Countercurrent Chromatography could be used as a highly efficient and promising method for the preparation of high pure CRMs of active compounds from natural products, because of its high loading capacity and good reproducibility.

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