ORIGINAL PAPER

Ming-Yong Xie · Lei Li · Shao-Ping Nie · Xiao-Ru Wang · Frank S. C. Lee

Determination of speciation of elements related to blood sugar in bioactive extracts from *Cyclocarya paliurus* leaves by FIA-ICP-MS

Abstract This paper describes the development and application of FIA-ICP-MS technique for the speciation of trace elements in extracts of *Cyclocarya Paliurus* (Batal.) Iljinsk (CP) leaves, an herbal tea used widely in China as dietary supplement for Elements Related to Blood Sugar (ERBS), which are reported to have the beneficial effects of lowering blood sugar. Most ERBSs are found to be water-soluble. On the basis of elemental distribution, the aqueous solutions of CP of different origins can be separated into two groups: one is rich in organo-magnesium, organo-manganese, organo-zinc, inorganic copper, and inorganic nickel; while the other is rich in inorganic magnesium, inorganic manganese, inorganic copper, inorganic nickel and organo-zinc. The ERBSs in two bioactive extracts of CP named EE and POLY were analyzed and compared, and the results suggest that the extracts are good dietary supplement for people, especially Diabetes Mellitus (DM) patients through the provision of ERBSs of Mn, Zn, Mg, Cu, and Ni in organic or inorganic forms.

Keywords Cyclocarya *paliurus* (Batal.) · Speciation · Reducing blood sugar · FIA-ICP-MS

Introduction

Cyclocarya Paliurus (Batal.) Iljinsk (CP) is a medicinal herb, which has been widely used in China both as drug formulations in TCM (traditional Chinese medicine) and as an ingredient in health foods or dietary supplements for trace elements. Both water and alcohol extracts from the leaves of CP were found to be bioactive in animal tests, having the therapeutic functions of reducing blood sugar and blood pressure, and anti-tumor. It is believed that the therapeutic properties of CP are related to the enriched concentrations of several trace elements in their leaves, among them the notable ones include Mn, Zn, Mg, Cu and Ni. As a group, these elements are named ERBS (Elements Related to Blood Sugar) [1]. It has also been hypothesized that these elements are readily transferred into the extracts of CP leaves along with the organic components because of their existence as metal-organic complex species [2]. However, systematic compositional and biochemical data are lacking to substantiate these claims. It is towards this goal that the current study was initiated.

This paper summarizes our results on the measurement and speciation of ERBS in CPs produced under different cultivation conditions. In recent years, the analysis and speciation of elements in health foodstuff and natural herbal medicines has become a subject of considerable interests because the bioactivities of the trace elements vary greatly with their chemical forms. Many reports have been published on the subject [3–12], and hyphenated FI-ICP-MS (Flow Injection-Inductively Coupled Plasma-Mass Spectrometry) technique represents one of the newest and most powerful techniques in such applications [9, 10]. By now, the hyphenated technology of FI-ICP-AES/MS system has been finding increasing applications in environmental and biochemical studies involving trace elements analysis. In this paper, the development of the FI-ICP-MS technique for the speciation of ERBSs in CP is also described.

M.-Y. Xie (✉) · S.-P. Nie
Key Laboratory of Food Science of Ministry of Education,
Nanchang University,
Nanchang 330047, People's Republic of China
e-mail: myxie@ncu.edu.cn
Fax: +86-791-8305826

L. Li · F. S. C. Lee
First Institute of Oceanography, State Oceanic Administration,
Qingdao 266061, People's Republic of China

X.-R. Wang
Key Laboratory of Analytical Science of Ministry of Education,
Xiamen University,
Xiamen 361005, People's Republic of China

Materials and methods

Instrumentation

Elemental analysis was performed on a PESCIEX ELAN 6000 ICP-MS (Perkin-Elmer, USA). The operating

Table 1 ELAN 6000 instrumental operating parameters

RF power	1000 W
Plasma	15 l/min
Auxiliary	0.8 l/min
Cross-flow nebulizer	0.85 l/min
Sampler/skimmer cones	Nickel
Scan mode	Peak hopping
Integration time per element	1 s
Short settling time	Enabled
Detector mode	Pulse counting
Quadrupole resolution	Normal (0.6–0.8 amu at 10% peak height)
No. of isotopes simultaneously monitored	1
Auto lens	On
Transient signal processing	Average value, 5-points smoothing

Table 2 FIMS-400 flow injection program for FIA-ICP-MS[a]

Step	Time (s)	Pump1 (rpm)	Pump2 (rpm)	Valve position	Remarks
Prefill	60	50	0	1	Prefill lines
1	5	80	0	2	Load
2	12	0	50	2	Wash
3	30	0	50	1	Elute
4	30	3	50	2	Wash
Post	—	25	0	1	Feed plasma

[a]Prefill: Flushes the tubing leading from the reagent vial with methanol; Step1: the sample is passed through the analytical column (4.0 ml/min); Step2: the tube is washed with Milli-Q water and the sample is eluted into the plasma; Step4: the valve is returned to the wash position to return the column for loading the next sample

conditions of the instrument are shown in Table 1. Model FIMS-400 Flow Injection Analysis (FIA) system used for ICP-MS analysis was operated according to the programme shown in Table 2. A MK-II microwave digestion system (Shinco, Shanghai, China) was employed for the digestion of samples. A STAR II system (CEM Co., USA) was used for the leaching experiments. IR analysis was carried out on a FT-IR instrument from Thermo Nicolet 370.

Standard reference materials of bush twigs and leaves (GBW 07602: GSV-1) were obtained from Chinese National Research Center for Certified Reference Material. Before use, all beakers and glassware were soaked in 20% HNO_3 of analytical-reagent grade for 48 h. All Teflon vessels were boiled for three times in 20% HNO_3. They were then washed with de-ionized water and ultra pure water before use.

Reagents

Methanol and HNO_3 were both of reagent grade. H_2O_2 was of analytical reagent grade; ultra pure water was pre-

pared by Milli-Q[50] (Millipore Corp., Bedford, MA) water purification system (>18 MΩ).

Standard solutions of multi-elements used for calibration were prepared by diluting the primary standard stock solution from Shanghai Institute of Material, Ministry of Machine Industry, P. R. China (1 g/ml each of K, Ca, Mg, Na, Fe, Zn, Li, V, Cr, Ni, Cu, Mn, Se, Sr).

Preparation of CP samples

Samples of CP leaves I and II were collected in Xiushui, and III, IV were collected from Jinggang Mountain, both in Jiangxi Province, China. Samples I and III were tender leaves, which were picked in May or June; whereas samples II and IV were old leaves picked in September or October of the same year. Before analysis, all samples were exposed to the sun for 6 h and then dried in oven at 103 °C for 4 h.

Crude polysaccharide was isolated from the powders of CP leaves by microwave-assisted extraction. The CP leaves (40 mesh) were first weighed and extracted with diethyl ether (30–60 °C) in a Soxhlet apparatus for 8 h to remove the color pigments. The diethyl ether in the extract solution was removed by evaporation and the dried extract was then placed in a microwave extraction tube. Water was added at a ratio of 1:8 W/V ratio of sample to water, and the solution was extracted in a microwave oven for 30 min at the temperature of 75 °C. The solution was cooled and centrifuged (3500 rpm) for 30 min to separate the supernatant and the residue. After decantation of the supernatant, the residue was extracted again with water (1:5 W/V ratio) in a microwave oven under the same conditions. The two supernatants were combined and concentrated at reduced pressure at 50 °C. After cooling to a temperature of 0–4 °C, 2.5 times of acetone was added to the concentrate and the solution was allowed to stand overnight in a refrigerator. The precipitates formed in the solution were collected and freeze-dried at liquid nitrogen temperature to yield yellow powders. The dried powder was then re-dissolved in pure water, centrifuged at 4000 rpm for 20 min, and the supernatant separated by centrifugation. The supernatant was further dialyzed for 36 h in natural water and 12 h in ultra pure water using a 1500-Da membrane. The acetone precipitation step was repeated to yield the PLOY fraction. FT-IR spectra using KBr technique showed the characteristics of POLY absorption bands (Fig. 1).

The ethanol extract (EE) of CP leaves was prepared by extraction of dried CP leaves (50–70 mesh) with 70% EtOH. A 1:8 (w/v) ratio of sample/ethanol was used for extraction. The mixture was allowed to stand overnight, and then reflux at 80 °C for 1.5 h. The extract was filtered through an 80-mesh filtrate cloth, and the residue was extracted again under the same conditions for another hour. The two extracts were combined, followed by ethanol removal (40 °C) and drying under reduced pressure to yield the brownish EE extract.
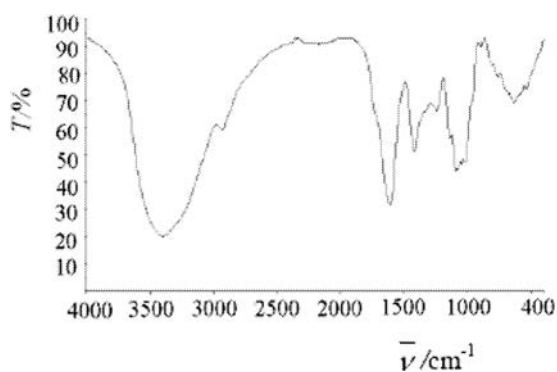
Fig. 1 FT-IR spectrum of polysaccharide fraction material from *Cyclocarya paliurus*

Fig. 2 Flow chart of speciation analysis

Microwave digestion

Approximately 500 mg sample of CP bush twigs and leaves and 300 mg of standard reference material were each accurately weighed into respective Teflon vessels, followed by the addition of 5 ml HNO_3. The vessels were capped and allowed to stand overnight for pre-digestion. The samples were then digested in the microwave heating system for 3 min at 1.52 MPa and 800 W power. They were then cooled to room temperature in a water bath. The microwave heating/cooling cycles was repeated twice. Afterwards, 2 ml H_2O_2 was added and the sample was again treated by microwave digestion under the same conditions as above. At the end of digestion treatment, the teflon inserts were removed from the bomb jacket and the digestion solvent was tipped out into a 50-ml-calibrated flask. The solution was diluted to the volume of 50 ml with 2 mol/l nitric acid, and then filtered through a 0.45-μm membrane for ICP-MS determination. Two-hundred milligrams of POLY or 300 mg of EE were also weighed and digested in the same way.

Microwave-assisted CP leaching

CP sample (1.5 g) was accurately weighted into quartz vessels and 80 ml ultra pure water was added. The vessels were placed inside the STAR II microwave system and heated under the pre-set program. The extraction conditions were programmed in two stages. In the first stage the system was heated rapidly to the designed temperature (100 °C) in 3 min. This is followed by constant temperature heating at 100 °C for 5 min. Then the vessels were cooled down to room temperature, and the extract was poured into a calibrated flask. Washed the quartz vessels with a little water three times. The solutions were then filtered through a 0.45-μm membrane filter for ICP-MS determination.

Speciation analysis and bioactivity testing

Polysaccharides and protein exist in nearly all plants. Some complex forms of polysaccharide were found to be bioactive, e.g., some polysaccharides from tea are known to be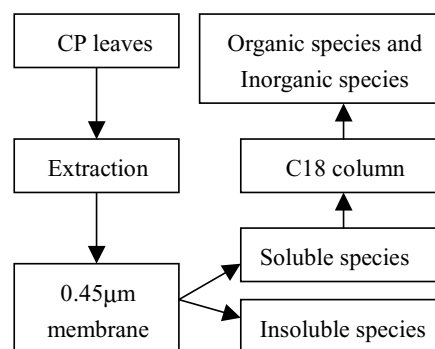 capable of reducing blood sugar. Some polysaccharides could exist as ligands in complex organometallic species. To confirm this, speciation analysis of trace metals was carried out according to the flow scheme given in Fig. 2. One hundred and eighty-two milligrams of dried POLY and 50 mg of EE samples were accurately weighed and each placed in a 100 ml flask along with 100 ml pure water. After standing overnight, the supernatant was filtered through 0.45 μm membrane filters. The solubles were further separated into organic and inorganic fractions by on-line FIA-ICP-MS using C-18 columns obtained from Alltech Inc. Diabetic mice model based on i.v. alloxan model was used to determine the bioactivity of POLY and EE [13]. Details of both speciation analysis and bioactivity testing can be found in the sections later.

ICP-MS analysis

ICP-MS was used for the analysis of ERBS. An isotope selection procedure was used to reduce interference caused by the isobaric elemental and polyatomic ionic species [14, 15]. Background interferences were corrected by subtracting the reagent blank signals, and the analytical blanks used in this paper is ultra pure water prepared by Milli-Q50 (>18 MΩ) mentioned above. External standards method was used for sample quantification. The standard reference material GBW07602 (GSV-1) was used for recovery study since GSV-1 is very similar in plant tissue and matrix to CP samples. The two also have similar distribution and concentrations of trace elements of K, Ca, Mg, Na, Fe, Zn, Li, V, Cr, Ni, Cu, Mn. The accuracies of the method as expressed by % recovery of individual elements ranged from 82.4% (Ni) ~113% (Al). The signal of the element Ni may be influenced by $^{44}Ca^{16}O$ and $^{43}Ca^{16}O^{1}H$ [14]. The precisions of the measurements are better than 10.8% RSD [14]. The ICP-MS instrument was controlled by ELAN 6000 software. In the extract of sample II, know concentrations of Mg, Mn, Cu, Zn and Ni were added. The spiked samples were then analyzed according to the same procedures as sample analysis. Recovery values were calculated based on standard addition method. The limits of detection (LOD) were calculated as three times the noise of blank signals, as listed in Table 3. Table 3 shows that recoveries for individual elements ranged from 92 to 103%, with

Table 3 Results from Detection Limits and recovery studies[a]

Element	LOD (μg/l)	Concentration before spike (μg/l)	Amount spiked (μg/l)	Concentration found after spike (μg/l)	Spike found (μg/l)	Recovery[b] (%) n=6, 3δ
Mg	12	1350±80	200	1540±100	190	95
Mn	2.9	109±2	50	155±5	46	92
Cu	0.4	33.3±2.7	20	52.6±2.2	19.3	96.5
Zn	1.2	31.4±2.2	20	52±2	20.6	103
Ni	0.15	3.58±0.13	20	22.5±1.3	18.9	94.6

[a]Results are expressed as the mean ± standard deviation (SD) for triplicate determination

[b]Derived from the ratio of the found spike value to the amount spike value

better than 8% RSD. The LOD of Mg, Mn, Cu, Zn, and Ni (n=6, 3δ) were 12, 2.9, 0.4, 1.2 and 0.15 μg/l, respectively.

Results and discussion

Optimization of speciation analysis procedures

In this study, the terms of "organic metals" or "Inorganic metals" are operationally defined. As shown earlier in Fig. 2, the "soluble species" after membrane filtration was further separated by C-18 column. In the paper [16] a method was established for the determination of classification of the inorganic and organic metals in Chinese tea infusion detected by multi-channels ICP-AES after C-18 column separation. In this system a synthesized complex of Zinc-rutin was used to demonstrate the ability of species separation of the column using proper solvents under the same operating conditions. The inorganic metals were first eluted from the column by water elution. On the other hand, the organic bound metals were retained in the column because of their hydrophobic nature. After the complete elution of inorganic metals, the retained organic metals were then washed from the column using organic solvent of methanol-contained nitric acid. The optimized operating conditions for the speciation analysis are given in Table 4.

According to the characteristics of the FIMS-400 system, a sampling velocity of 5.0 ml/min was selected to ensure that appropriate amount of sample could be sampled in the FIA sample introduction step. In the meantime, the type of eluting solvents and the eluting time were optimized in order to achieve the best separation efficiency [15]. The elution time of water for the inorganic metals was 15–30 s; and the elution time of acidic solvent used for organic metal elution, i.e., 5% methanol in nitric acid, was 15–40 s, respectively. As an example, the nickel elution profiles are shown in Fig. 3. The First peak was inorganic
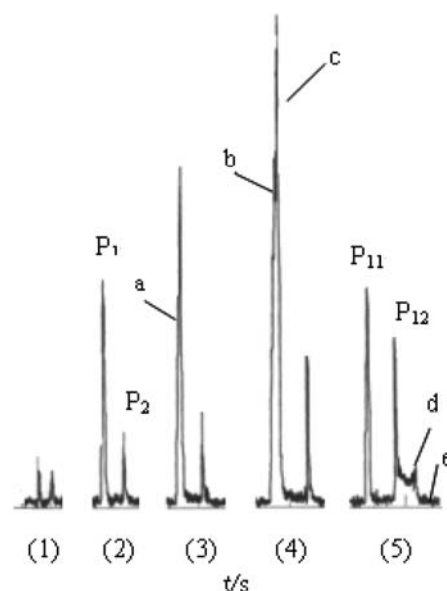

Fig. 3 Ni speciation at different separation conditions in CP extract. (1), (2), (3), (4), (5): separation conditions; P1, P11: inorganic species; P2, P12: orgranic species; a, b, c, d, e: the shape of various peak

nickel and the second peak was organic nickel. The first elution profile shown on the very left is with 3 s sampling. The next run was of 10 s sampling, and adequate signal strength was obtained. In runs 3 and 4 with 15 and 30 s sampling, respectively, the appearance of shoulder peaks suggest sample over loadings. Run 5 was for 10 s sample after the C-18 column had been used after 50 cycles; and the figure shows that good separation of inorganic and organic species can still be obtained. There was a drop in the column efficiency after 50 cycles of runs, but the recovery of the elements was still between 90 and 110%. In general, C-18 column was stable within pH 3–9, but the column efficiency would be lower if the elution solvent is too acidic (pH lower than 3.0).

Distribution and speciation of metals in CPs

The results showed that the CP leaves are very rich in ERBSs (Table 5). The concentrations of detected elements including ERBS are K, Ca, Mg, Mn, Fe, Zn, Cu, Ni, Cr, Se, V and Li. Of these elements, Mn and Zn are the two

Table 4 optimizations of speciation analysis

Item	Parameters
Sampling velocity	5.0 ml/min
Sampling time	10 s
Mixture eluting solvent	Nitric acid contained 5% (v/v) methanol
Water eluting	15–40 s

Table 5 Elemental concentrations in CP Leaves[a]

Element	I	II	III	IV	Range
Li	0.25±0.03	0.42±0.02	0.100±0.001	0.080±0.002	0.08–0.42
K	22000±800	16100±400	19200±500	12700±300	12700–22000
Mg	3760±160	2590±130	3760±140	2410±110	2410–3760
Mn	718±88	712±58	716±25	715±46	712–718
Zn	66.8±2.9	67.3±3.2	67.7±4.6	68.5±2.4	66.8–68.5
Se	1.22±0.86	0.56±0.01	0.97±0.03	2.07±0.44	0.56–2.07
V	4.23±0.28	5.38±0.13	3.75±0.15	3.81±0.13	3.75–5.38
Cr	1.31±0.16	1.72±0.24	1.39±0.14	1.34±0.12	1.31–1.72
Ni	3.96±0.13	2.63±0.18	4.18±0.31	1.70±0.34	1.70–4.18
Cu	40.6±0.5	33.5±0.5	29.6±0.7	24.2±0.20	24.2–40.6
Ca	15700±200	14200±200	16500±200	21000±200	14200–21000
Fe	254±5	349±8	102±5	120±4	102–349

[a]Results are expressed as the mean±standard deviation (SD) for triplicate determinations. The unit is mg/kg for all elements. I, tender leaves from Xiushui district; II, old leaves from the same district; III, tender leaves from Jinggang Mountain; IV, old leaves from the same district

representative ones in CP, because the contents of the two elements are 3–10 times higher than those commonly found in extracts of plant origins [17]. Furthermore, the concentrations of the two elements are fairly constant among CPs grown at different locations. This is especially true for Zn, as can be seen in the data listed in Table 5.

Speciation results for the five elements Mg, Mn, Cu, Zn, and Ni in the water extracts of samples I, II, III and IV originated from different regions and collected at growing periods are shown in Table 6. Results show that the analytical recoveries of the speciation technique range from 90.2–98.9%. For all samples, the total soluble metals accounts for more than 96.8% of the total metals in the samples, with only less than 3.2% of insoluble metals. In general, the organic metals account for a major fraction or majority of the total metals. In sample I, the concentrations of the soluble and organic metals decrease in the order of Mg, Mn, Zn, Cu and Ni. On the other hand, the inorganics in decreasing order are Mg, Mn, Cu, Zn and Ni. The order of Cu and Zn in the two orders is reversed. Sample III results show recovery values ranged from 93.9 to 98.2%. Different from sample I, the concentration order of the five elements in both the organic and the inorganic fractions are the same, i.e., Mg>Mn>Zn>Cu>Ni. This could be due to the high concentration of total soluble Zn in sample III. The recoveries observed in sample IV ranged from 90.2 to 99.1%, and its metal distribution is similar to those of III.

When CP samples from different areas are compared, Table 6 shows that other than Mg, the soluble elements in extract of sample II are higher than those of IV. Mg in sample IV, however, is 2.5 times those of II. Since the organic magnesium accounts for only 25.1% of the total soluble, its concentration in sample IV is only 13.6% higher than those of sample II. All five elements in sample II are proportionally higher in organic forms. Thus, taking sample II is beneficial in terms of having more enriched ERBSs in organic forms.

Sample III contains higher soluble ERBS elements, especially organic zinc, than sample I. Sample I, however, is higher in organic magnesium, manganese and copper. There is no noticeable difference in Ni, which is low in either case. Thus, as far as tender leaves are concerned,

sample III offers more soluble ERBS elements and organic zinc, whereas sample I offers higher organic magnesium, manganese and copper.

When CP leaves from the same area are compared (sample I vs. II), Table 6 shows that sample II contains higher soluble Mg, Mn and Zn, organic manganese and organic nickel, but lower organic magnesium and soluble nickel and copper. There is no marked difference in organic copper and zinc. When samples IV and III are compared, the former is higher in soluble magnesium and manganese, but lower in other metals. Thus, in daily consumption, drinking CP tea of old leaves would take in higher soluble magnesium and manganese; while drinking tender leave CP tea would take in more organic zinc, copper and soluble nickel and copper. All CP teas are enriched in soluble zinc, and their beneficial effect in supplement ERBS elements is significant.

We now illustrate further the characteristics of ERBS elements in terms of their difference in speciation. For Mg, sample IV contains the highest soluble forms, whereas sample I contain the lowest. On the other hand, sample I is enriched with the highest organic ERBSs. The organic magnesium in sample I accounts for the highest percentage in soluble metals. Sample II contains the highest soluble Mn, and the highest organic manganese concentration. Although sample I has the lowest soluble Mn, it has the second highest amounts of organic manganese among all samples studied. In fact, a person would have sufficient Mn supply if he/she consumes 800–1000 ml of CP tea from sample II. One thousand milliliters of CP tea would supply 2.63 mg of Mn, of which 1.57 mg are the organic ones. Sample IV contains the lowest soluble and organic copper. Both Sample I and III contain higher soluble Cu but sample I is more enriched in organic copper. Thus, from the viewpoint of Cu bioavailability, sample I would be preferred. Sample III contains noticeably higher Zn and Ni than the other CP samples, and the highest organic zinc. In summary, it is seen that the distribution of ERBSs in CP varies with their geographic origin. Sample from Xiushui (samples I and II) are enriched in organic magnesium, manganese and zinc, whereas CP leaves from Jinggang Mountain (sample III and IV) are more enriched in inorganic magnesium and manganese.

Table 6 Concentrations of Mg, Mn, Cu, Zn, and Ni in different sample of CP leachate[a]

Element	Concentration in original extract (μg/l)	Soluble species (μg/l)	Organic species (μg/l)	Inorganic species (μg/l)	Recovery (%)[b]
Sample I: Tender leaves from Xiushui					
Mg	6020±380	5990±410	4320±220	1540±60	97.8
Mn	1480±50	1470±90	1090±60	277±10	93.2
Cu	75.5±3.2	74.7±2.5	30.6±1.6	42.8±1.6	98.3
Zn	146±7	145±6	106±5	37.0±1.9	98.9
Ni	9.75±0.51	9.68±0.42	1.70±0.65	7.22±0.36	92.1
Sample II: Old leaves from Xiushui					
Mg	6500±320	6440±210	3600±170	2680±150	97.5
Mn	2650±110	2630±110	1570±50	936±40	95.3
Cu	69.7±2.5	69.1±2.3	30.1±1.1	38.1±1.9	98.7
Zn	201.5±10.4	199±10	109±4	88±4	99.3
Ni	6.76±0.31	6.71±0.32	2.81±0.12	3.35±0.14	91.8
Sample III: Tender leaves from Jinggang					
Mg	8320±400	8180±560	3210±390	4820±390	98.2
Mn	1640±80	1630±80	697±29	885±47	97.2
Cu	78.4±2.9	77.6±2.5	25.2±1.1	50.5±3.2	97.6
Zn	380±26	372±22	251±19	107±9	96.2
Ni	11.3±0.4	11.1±0.3	1.59±0.10	8.81±0.51	93.9
Sample IV: Old leaves from Jinggang					
Mg	16800±1380	16300±1040	4090±190	11700±760	96.9
Mn	2030±110	2010±80	674±30	1240±60	95.2
Cu	52.4±2.2	52.1±1.7	18.2±0.7	33.1±1.5	98.5
Zn	182±9	180±8	89.6±3.2	88.8±3.5	99.1
Ni	3.77±0.08	3.74±0.11	1.30±0.04	2.07±0.04	90.2

[a]Results are expressed as the mean ± standard deviation (SD) for triplicate determination

[b]The sum of organic species and inorganic species as a wt percentage of the total soluble species

Bioactive extracts of CP

The CP is very rich in POLY material (9.6 wt %). The FT-IR spectrum of the POLY extract is given in Fig. 1, which shows the characteristic polysaccharide spectrum. The metals are highly enriched in both polysaccharide and EE fractions, the two functional extracts of CP commonly used as dietary supplement for ERBSs. The concentrations of Cu, Zn, Se, and Ni in EE are higher than that in polysaccharide (Table 7), i.e., the concentrations of Copper, Nickel and Zinc in EE are, respectively, 13, 2.3, 1.5 times than that of POLY. The results from the speciation of the five elements in POLY and EE are shown in Table 8. As seen, the metals are predominantly organic species from our analysis.

The effects of POLY and EE on blood sugar value of DM mice are shown in Table 9. There was no significant difference in blood sugar values among DM mice before having CP extract (Ig) but the value of no control (NC) group was lower ($P<0.05$). The mice of POLY, EE and Xiaokewan (XKP) groups exhibit significant difference ($P<0.05$) from the DM control group (DMC) after having 4 weeks of Ig. This indicates that the POLY and EE ma-

Table 7 Concentrations of 12 ERBS in POLY and EE fractions[a]

Element	POLY	EE
Li	0.83±0.04	0.37±0.02
K	67500±1030	40312±1015
Mg	17050±280	3210±110
Mn	1630±40	257±12
Zn	56.1±1.3	83.3±2.8
Se	0.58±0.03	1.08±0.05
V	6.3±0.3	1.23±0.06
Cr	3.04±0.12	1.39±0.07
Ni	3.33±0.17	7.53±0.38
Cu	6.51±0.33	85.4±1.4
Ca	4360±200	979±20
Fe	402±12	34±2

[a]Results are expressed as the mean±standard deviation (SD) for triplicate determinations. The unit is mg/kg for all elements. POLY: complex carbohydrates; EE: ethanol extract

terials had similar bioactivity of reducing blood sugar, and the activity was better than XKP after 2 weeks' experiment according to the measured blood sugar value. The results

Table 8 Speciation of Mg, Mn, Cu, Zn, and Ni in functional extracts of POLY and EE fractions[a]

Element	Concentration in extract (μg/l)[b]	Soluble species (μg/l)	Organic species (μg/l)	Inorganic species (μg/l)	Recovery (%)[c]
POLY					
Mg	30980±2540	28810±1610	11000±680	17380±1010	98.5
Mn	2970±180	2790±110	1470±60	1250±60	97.3
Cu	11.8±0.3	11.3±0.4	6.57±0.20	4.43±0.19	97.1
Zn	102±4	97.9±2.8	63.0±2.8	33.5±1.4	98.6
Ni	6.0±0.3	5.7±0.2	2.01±0.13	3.38±0.12	94.5
EE					
Mg	1600±90	1350±80	583±41	740±44	98.2
Mn	128±5	109±2	61.3±4.2	45.7±1.4	98.1
Cu	42.7±1.4	33.3±2.7	26.8±1.1	5.6±0.2	97.2
Zn	41.7±2.2	31.4±2.2	19.1±1.2	12.1±0.4	99.4
Ni	3.77±0.10	3.58±0.13	1.91±0.06	1.47±0.34	94.3

[a]Results are expressed as the mean±standard deviation (SD) for triplicate determinations .The unit is mg/kg for all elements
[b]Calculated by the concentration of POLY solution(1.8 mg/ml) and the EE solution (500 μg/ml)
[c]Derived from the ratio of the sum of organic species and inorganic species to soluble species

Table 9 Blood sugar value in serum of experimental mice (mmol/l)[a]

Group	Dose	Before Ig	Ig 1 week	2 weeks	4 weeks
NC		3.79±5.68 (15)	4.0±1.2 (15)	5.9±1.7 (15)	4.2±1.0 (14)
DMC	70 mg/kg per day	21.5±5.1[b] (17)	17.2±7.4[b] (15)	19.2±7.2[b] (13)	15.5±9.1[b] (12)
POLY	32 g/kg per day	20.8±4.6[b] (16)	17.6±9.1[b] (16)	11.8±3.8[c] (13)	9.1±1.6[c] (11)
EE	32 g/kg per day	19.3±4.5 (17)	18.7±9.9 (16)	15.4±6.4 (11)	9.9±3.9[c] (10)
XKP	2.5 g/kg	23.6±5.4[b] (17)	13.7±6.1[b] (16)	12.6±4.7[c] (11)	9.9±3.9[c] (10)

[a]Animal amounts
[b]Compare to no control: $P<0.05$
[c]Compare to DM mice: $P<0.05$; number in parentheses shows the number of experimental mice

are still preliminary and further animal experiments are in progress for confirmation.

Conclusion

The elemental concentrations and speciation in CP and its infusions and extracts are reported in this paper. An analytical scheme was developed for the speciation of ERBS (Mn, ZN, Mg, Cu and Ni) in CP and their extract samples. The elements were fractionated into four fractions: soluble vs. particulate-associated fractions by 0.45 μm membrane filtration; and organic vs. inorganic fractions of the soluble fraction using C-18 bonded silica column. Major metal species in each fraction were determined by on-line FIA-ICP-MS. Preliminary animal experiments confirm the claims that the trace elements of ERBSs are beneficial in lowering the blood sugar in mice. Further animal experiments and bio-testing are in progress to provide deeper insight into the nutritional significance of CP and ERBSs.

References

1. Li Lei, Xie Mingyong, Yi Xing (1999) Review on the relationship between speciation and its blood sugar reduction activity. Trace Elements Health Res 16(3):69–71
2. Xie Mingyong, Li Lei (2001) Review studies on chemical constituents and bioactivities of *Cyclocarya paliurus*. Chin Tradition Herbal Drugs 32(4):365–366
3. Ye Yu-qong, Deng Quan-Yao (1994) Study on the analyses of inorganic and organic Speciation of Fe, Cu, Mn, and Zn in aqueous tea immersing. J Sichuan Univ (Nat Sci Edn) 32(3):329–331
4. Xue Han-bin, Laura Sigg (1994) Zinc speciation in Lake Waters and its determination by ligand exchange with EDTA and differential pulse anodic stripping voltammetry. Anal Chim Acta 284:505
5. Pitts L, Fisher A, Worsfold P, Hill SJ (1995) Selenium speciation using high-performance liquid chromatography-hydride generation atomic fluorescence with on-line microwave reduction. J Anal Atom Spectrom 10:519–520
6. Lin Jianming, Yang Penyuan, Wang Xiaoru (1994) Simultaneous analysis of multi-element speciation in tea extract. Anal Lab 13(3):6–8
7. Warren RLC, Stephen JH, Les E (1996) Directly coupled high performance liquid chromatography-inductively coupled plasma-mass spectrometry for the determination of organometallic species in tea. Micro Chem J 54:88
8. Shao Li-jun (1999) Application of flow injection analysis with inductively coupled plasma-atomic emission spectrometry combined technology in bio-sample analysis. Chem World 6:288
9. Denoyer ER, Stroh A, Lu QH (1993) Characterization of operating parameters in flow injection ICP-MS. Atom Spectrosc 14:55

10. Debrah E, Alary JF (1999) Improved sample throughput using flow injection rapid microsampling for ICP-MS. Atom Spectrosc 20(1):1–5
11. Xie MY, Messerschmidt J, von Bohlen A, Ma YH, Pfeilsticker K, Günther K (1995) Selengehalte in Tee aus verschiedenen Provinzen der Volksrepublik China. Z Lebensm Unters Forsch (Eur Food Res Technol) 201:303–306
12. Xie MY, von Bohlen A, Klockenkämper R, Jian XH, Günther K (1998) Multielement analysis of Chinese tea (*Camellia sinensis*) by total-reflection X-ray fluorescence. Z Lebensm Unters Forsch (Eur Food Res Technol) 207:31–38
13. Yi Xing, Xie Mingyong, Wen Huiliang (2001) Hypoglycemic effects of Cyclocarya Paliurus on alloxan diabetic mice. Nat Product Res Dev 13(3):52–54
14. Li Lei, Xie Mingyong, Wu Xihong, Sun Zhenhua, Wang Xiaoru (2000) Nutrition elements determination in *Cyclocarya paliurus* and its extract by MAP-ICP-MS. Food Sci 21(2):53–57
15. Li Lei, Xie Mingyong, Sun Zhenhua, Wu Xihong, Sun Dahai, Wang Xiaoru (2000) The studies on the elements transferring characteristics and element speciation in the extract of *Cyclocarya Paliurus* leaves. Chem J Chin Univ 21(5):707–709
16. Lin Jianming, Zhuang Zhixia, Yang Pengyuan (1993) Multielement speciation in tea by FIA-ICP/AES. J Instrum Anal 12(6):49
17. Wang Kui (1998) (eds) Handbook of trace element analysis and data in life science. Chinese Metrology Press, Beijing